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A REGENERATION STUDY WITH EMBRYONIC TISSUES
AND EMBRYONIC CELLS OF CARROT, DAUCUS CAROTA L.
AND APPLE, PYRUS MALUS, L.

Submitted by
Athanasios C. Rubos
for the degree of Ph.D. of the
University of Bath
1983

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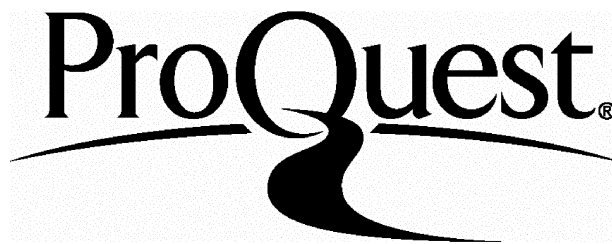
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List of abbreviations

IAA	=	indole acetic acid
IBA	=	indole butyric acid
2,4-D	=	2,4-dichlorophenoxyacetic acid
NAA	=	naphthalenacetic acid
6BAP	=	6-benzyladenine (benzyl amino purine)
Nitro-BT	=	Nitro blue tetrazolium
Approx.	=	approximately
Diagr.	=	diagram
Fig.	=	figure
Cv.	=	cultivar
Var.	=	variety
PCA	=	point cotyledons arise

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Abstract

A method for isolating embryonic cells in order to establish primary cell cultures has been developed. Embryos, intact or cut into small pieces, were digested with different cell-separating enzymes and after 24h post-digestion in 0.6M mannitol they were sheared by using a hypodermic syringe. The resulting cell suspension consisted of morphologically intact cells. This work has been done using embryos of species belonging to widely differing families to show the general applicability of the method. In the procedure to macerate embryos into single cells it was found that embryos were surrounded by a cuticle.

Primary cultures of embryonic cells of carrot, Daucus carota L., var. Chanteney Red Core and apple, Pyrus malus L., var. Phiriki and Golden Delicious, were established. Carrot embryo cells divided and approximately 30% of them gave direct growth to embryoids, while approximately 40% formed callus and 20% showed increase of their size when cultured in the Fujimura and Komamine (1975) agar medium. Apple embryo cells cultured in variously supplemented agar media showed only a few divisions but no colony formation was observed. In liquid cultures apple embryonic cells divided and showed the typical sigmoid curve. When they were transferred to low auxin-containing or auxin-deprived media they showed rare embryo formation.

Adventitious roots, shoots or both and embryos were formed in apple embryonic tissues of the varieties Phiriki and Golden Delicious when cultured under light and at $25 \pm 2^{\circ}\text{C}$ on the Murashige and Skoog (1962) medium as modified by Pech et al. (1975) containing various

growth regulators or combinations of them. Of the different parts of the embryo tissue, the cotyledon was most responsive in morphogenesis. Embryos developed from structures formed mainly at the base of the cotyledon. The majority of these structures proliferated and formed callus, while very few of them continued their growth as embryos. The formation of shoots and roots was also localised at the base of the cotyledon. Adventitious shoots when transferred to the same medium containing 2mg/l IAA, rooted and formed plantlets.

CHAPTER 1
GENERAL INTRODUCTION

GENERAL INTRODUCTION

In this work the isolation of embryonic cells was attempted^{emp} in order to establish primary cell cultures. Maceration of embryos of carrot and barley as representatives of monocotyledons and dicotyledons respectively, and maceration of embryos of various other herbaceous and woody species was performed in order to establish a method for embryonic cell isolation with general applicability.

Cell cultures derived from zygotic or tissue culture embryos are of particularly high embryogenic potential (Steward et al., 1964; Vasil et al., 1964; Konar and Nataraja, 1965). Therefore embryonic tissues have been used as a possible approach to culture of species not responding to morphogenetic stimuli e.g. cereals and others (Ammirato and Steward, 1971; Button et al., 1974; Cummings et al., 1976; Gosh and Galum, 1978; Green and Phillips, 1975; Radojevic et al., 1975; Shimada, 1978; Thomas et al., 1977; Vasil and Hildebrandt, 1966; Vasil and Vasil, 1981).

Although cell cultures originating from embryonic tissues contain a high morphogenetic capacity, like the secondary cell cultures of different origin, they show the disadvantage of cell aggregation, and a lack of uniformity of cell types (Melchers and Bergmann, 1959; Tulecke, 1966; Liau and Boll, 1971).

An ideal cell culture for embryogenetic studies could be an aggregate-free population of true meristematic cells. Such cell populations should be obtained by macerating either embryos at different stages of their development or meristems. So far the most attractive source of free cell inocula isolated directly from the

plant body has been mainly the mesophyll tissue (Coutts and Wood,1975; Jullien and Rossini, 1977; Miksch and Beiderbeck ,1976; Servaites and Ogren, 1977; Pelcher et al.,1974 ;Schwenk,1980 and Takebe et al.,1968).

After a successful regeneration study had been established with primary cell cultures of embryo origin, techniques could then possibly be transferred to cells originated from clonal mature tissue, e.g.mesophyll or meristem. Cell culture of clonal material coupled with plant regeneration would be a very useful tool for the study of the somaclonal variation with respect to plant improvement and possibly for the asexual propagation of certain clones.

The genetic variability occurring within cell cultures has been demonstrated for many species (D'Amato, 1978; Challeff and Keil,1981; Schaeffer and Sharpe, 1981). It can be concluded from a review of the literature that tissue and cell cultures are frequently rich and novel sources of genetic variability or somaclonal variation (Larkin and Scowcroft, 1981).

The potential usefulness of somaclonal variation for plant improvement first became apparent in sugar cane. Variation in morphological (Liu et al.,1972); disease resistance (Nickell and Heinz, 1973; Heinz et al.,1977; Larkin and Scowcroft, 1981), cytogenetic and isozyme traits (Larkin and Scowcroft, 1981) has been observed amongst sugar-cane somaclones. Shepard et al., (1980) argued that it could be simpler to selectively improve popular potato varieties than to create new ones and they claim to have demonstrated this by the use of somaclonal variation. Larkin and Scowcroft (1981) gave substantive and extensive examples of somaclonal variation in culture subclones and in regenerated

plants (somaclones). They also discussed a number of possible mechanisms for the origin of this phenomenon and argued that this variation is already proving to be of significance in plant improvement.

The major limitation with regard to the full exploitation of somaclonal variation and in vitro selection techniques for plant improvement is that of the dependable plant regeneration from cell cultures of many crop species (Thorpe, 1982). The success of in vitro selection for many agriculturally important traits in tobacco (Challeff, 1980; Challeff and Kiel, 1981; Berlyn, 1980) is because plant regeneration from a range of genetic backgrounds is well established. Successful experiments combining single-cell cloning techniques with efficient plant regeneration systems yielding populations of cell-culture-derived plants were recently reported for Egyptian henbane (Wernicke et al, 1979), pearl millet (Vasil and Vasil, 1980) and potato (Ayers, 1981). Lack of success in economically important crop plants, like soybean (*Glycine max* L.) is because failure of repeatable plant regeneration (Beaversdorf and Bingham, 1979; Phillips and Collins, 1982) has restricted the research effort to regeneration per se rather than on the wider aspect of in vitro selection.

A species could possibly be improved through somaclonal variation only if it is amenable to in vitro manipulation for the growth of cell cultures and for the easy regeneration of plants from variant cell cultures (Tomes and Swanson, 1982). For species not responding to morphogenetic stimuli and showing a repeatable lack of regeneration, it may be that primary cell cultures of zygotic embryo origin could constitute the intermediate step for successful regeneration studies from cell cultures originated from clonal mature tissue, although it is not known whether the same type of genetic variability would occur in such cultures.

Carrot was selected for studying primary cell cultures of zygotic embryo origin. It was selected as a representative of herbaceous species and because it is well known to be embryogenetically responsive. The purpose of using carrot first was to develop a model for primary cell cultures of other species.

The culture of carrot zygotic embryo cells was based on striking examples of totipotent non-zygotic development from free somatic cells (Steward and Mapes, 1963; Steward et al., 1963) and on the extensive literature on factors controlling embryogenesis in tissue and cell cultures. Reference to this literature is made in the following chapters concerning the work with carrot.

Various examples drawn from different species belonging to various families show that free somatic cells of many angiosperm species can be made to grow in isolation, and, if they do so and receive the correct balance of nutrients and stimuli, they may be brought into the state in which they will organize into plantlets. (Steward et al., 1966). Based on this idea and after a successful regeneration study with primary carrot embryo cultures had been established, the culture of free apple embryonic cells was attempted in order to induce morphogenesis and possibly plant regeneration.

Because zygotic apple embryo cell culture could not be based on similar work with apple morphogenesis, emphasis was given to the requirements of the embryonic tissues in respect to morphogenesis and plant regeneration. Knowing the factors that control embryogenesis or any other type of morphogenesis in apple embryonic tissue cultures, the induction of morphogenesis in embryonic cell cultures could be attempted on the idea that factors controlling morphogenesis in a certain

tissue and in primary cell cultures derived from the same tissue do not vary considerably.

The factors controlling morphogenesis and plantlet regeneration in apple embryonic tissue cultures were combined with the techniques applied to primary cultures of carrot embryo in order to induce morphogenesis and plantlet regeneration in primary cell cultures of zygotic apple embryo origin.

Apple, a woody deciduous fruit species growing in every European country, occupies a prominent place among the species of the family of Rosaceae. Multiple shoot proliferation cultures of apple have been used successfully for propagating both fruit and rootstock cultivars and this whole literature has been reviewed by Lane (1982).

Apart from the research work in the field of rapid shoot multiplication there is a considerable amount of research work concerning morphogenesis in apple tissue cultures. Mu et al., (1977), reported the induction of callus from apple endosperm and the differentiation of the endosperm plantlet. The aim of their work was to provide triploid and tetraploid materials for breeding work in agriculture and fruit cultivation. Mehra and Sachdeva (1979) raised calluses from sterile seeds, cotyledons, embryos, seedlings, roots, hypocotyls, stem, shoot tips, leaves and fruit tissue of apple in order to induce plantlet formation. Differentiation of isolated roots, leaves and shoots was observed from calluses of different parts of the seedling but not from the fruit callus. However the differentiation of whole plants was not obtained from any of the calluses. Organogenesis of Malus (Rootstock M.9) was reported by Wei-lun et al., (1979). In their work adventitious

shoots were obtained through callus which consequently rooted and apple plantlets were obtained.

The first studies on the culture of apple anthers were reported by Nakayama et al. (1971) and Nakayama et al. (1972). These investigators obtained a callus from cultured anthers in which roots were initiated. Later Kubicki et al. (1975) and Milewska-Pawliczuk and Kubicki (1977) announced the induction of embryoid development from pollen grains. The induction of androgenesis in vitro reported by the previous workers was the first report on adventive embryogenesis of apple. The next and the most recent report was by Eichholtz et al. (1979), who initiated adventive embryos of Golden Delicious apple from micropylar halves of nucellus.

While there is a considerable amount of research work on the micropropagation of apple by shoot multiplication and on morphogenesis in tissue culture of apple, the use of cell cultures of apple has been very limited and it is confined to the study of some metabolic aspects. The only exception is the previously reported induction of androgenesis in vitro from pollen grains.

The first reports concerning the growth characteristics of cell suspension culture of apple of fruit parenchyma origin appeared by Pech and Fallot (1974) and Pech et al. (1975). Since then apple cell cultures, always of fruit parenchyma origin, have been used for the study of catechol oxidase activity (Volk et al., 1978; Volk et al., 1979) and for the bioconversion of the salts of fatty acids and aldehydes into volatile components of the flavour of the fruit and ethylene production (Lieberman et al., 1979). Although apple cell cultures have been used for biochemical and physiological (Pech et al., 1975;

Wallner 1977) studies, they have been no reports of their having been used for morphogenetic studies.

From the above quoted literature on apple morphogenesis it is obvious that apple tissues can respond to morphogenetic stimuli. If culture of embryonic tissues and embryonic cells of apple could be proved to be successful in respect to morphogenesis and plant regeneration, then these techniques might be applicable to cells derived from mature clonal material. If plant regeneration could repeatedly be achieved from cell cultures originating from mature clonal material, then somaclones could be obtained as a possible source of variation for apple improvement.

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CHAPTER 2

ISOLATION OF CELLS FROM PLANT EMBRYOS

A. INTRODUCTION

The purpose of this study was to develop a method for isolation of single, morphologically intact and viable embryonic cells at densities high enough to make possible the establishment of either agar or liquid primary cell cultures. The first step in this work was to find an easy way for the isolation of intact embryos at a satisfactory rate particularly from those plant species which present certain difficulties either because of the small size of the embryo (carrot) or because of the existence of a hard woody endocarp (Pistacia terebintha). The second step was to break the isolated embryos to the single cells of which they consist, causing the least possible morphological damage to them and without seriously affecting their viability. For accomplishing the second step enzymic maceration with cell-separating enzymes (i.e. pectinases) was employed in the method and it was followed by a mechanical effect (shearing) which resulted in the release of single morphologically intact and viable cells from macerated embryos.

B. MATERIALS AND METHODS

a. Isolation of cells from carrot embryos

a-1. Maceration of carrot embryos with Macerozyme

Carrot seed var. Chanteney Red Core was soaked in water for 24h. The embryos were squeezed out in water with the help of a dissecting stereoscope, tweezers and a scalpel. Isolated embryos were digested for 2h at 32°C in 2ml of 1% Macerozyme (from Rhizopus species, Yacult Biochemicals Co. Ltd.) solution containing 0.6M mannitol (pH 5.7-5.8). During digestion the embryos were rotated at the low speed of 50rpm. After digestion they were rinsed with 0.6M mannitol solution and postdigested in 1.5ml of the same solution for 24h. Postdigested embryos were sheared several times using a 5ml glass hypodermic syringe with a number VI needle. After shearing the resulting cell suspension was poured through a double 45µm steel mesh fixed in a Millipore filter holder [Millipore (U.K.) Limited, Millipore House, Abbey Road, London NW10 7SP] to remove debris and any cell aggregates. Cells contained in the 1.5ml solution of 0.6M mannitol were counted with the use of a haemocytometer at 160 magnification.

For the digestion and postdigestion of the embryos 10ml glass vials covered with plastic tops were used.

a-2. Maceration of carrot embryos with other cell-separating enzymes

In addition to the Macerozyme isolated carrot embryos were also digested for 2h at 32°C in solutions of varying concentrations of the following enzymes: 1) Macerase, an enzyme mixture from

Rhizopus species (Calbiochem). 2) Rohament P a pectin glucosidase from Aspergillus species either alone or in combination with the 3) Cellulase präp 223oA (both products of Röhm GmbH) and 4) Pectinase SS from Aspergillus niger (Yacult Biochemicals Co. Ltd.). The pH of the above solutions was adjusted respectively to 5.7, 5.0, 5.0 and 4.3. After digestion the embryos were treated as previously described in a-1 and the number of cells released per embryo was counted.

b. Isolation of cells from embryos of other species

b-1. Plant species

Other plant species from which embryos were macerated were the following: Phaseolus vulgaris var. Canadian Wonder, Pisum sativum var. Onward, Brassica oleracea var. Coleslaw, Lactuca sativa cv. Flucos, Apium graveoleus var. Solid White, Triticum vulgare var. Maris Huntsman, Hordeum vulgare var. Maris Otter, Malus domestica var. Phiriki and var. Golden Delicious, Olea europaea var. Chondrolia Chalkidikis and var. Amphissis, Pistacia terebintha, Prunus mahaleb, Prunus amygdalus var. texas, Prunus instititia and Citrus limonia of varieties introduced from Cyprus. Embryos from plums, cherries and apricots purchased from the local market were also macerated.

Bean, pea, cabbage, lettuce and celery were selected because they represent widely cultivated horticultural species and wheat and barley were selected as representatives for monocotyledons. Of these two varieties of apple Golden Delicious was used in this work because it constituted an easy source of seed isolated from apples purchased from the local market. A more detailed description of the var. Phiriki together with an explanation of the reasons for using

this variety are given in the introduction of Chapter 4. The varieties of olive *Chondrolia Chalkidikis* and *Amphissis* were selected because they represent two of the main varieties cultivated in Greece. The first occupies the coastal area of Macedonia and the second is cultivated in Thessaly, major part of Sterea Hellas, southern Epirus and north Peloponnese. *Pistacia terebintha* is a deciduous tree with strong resinous scent. In Greece it is used almost exclusively as a rootstock for Pistachios because it is very resistant to high and excessive concentrations of calcium in the soil and very resistant to very dry soil and atmospheric conditions. It shows **relatively** vigorous growth even when cultivated in very poor gravelly soils (Raptopoulos, 1970). *Prunus mahaleb* is used as an ornamental tree and as a rootstock for the *Prunus cerasus* showing very good compatibility. The fruits of this tree are used by confectioners and it is the species from which cherrywood pipes are made. *Prunus instititia* which is also known as wild plum and includes a wide range of genotypes, is used as a rootstock for apricots, cherries, plums and peaches. A more detailed description about *Prunus mahaleb* and *Prunus instititia* is given by Sficas (1978). *Prunus amygdalus* var. *texas* is a widely cultivated variety of almond now giving place to other varieties satisfying better the needs of the growers and of the market. Its open pollinated seeds are used for the production of rootstock material (seedlings) for almond varieties. *Citrus limonia* was selected as a representative from *Citrus* species which are so important for the horticultural industry.

The olive stones of the varieties *Amphissis* and *Chondrolia Chalkidikis*, the seed of *Prunus mahaleb* and *Prunus amygdalus* var. *texas*

were a gift of the Laboratory of Biology of Horticultural Plants of the University of Thessaloniki, Greece. Wheat and barley were supplied by National Seed Developmental Organization and some of the seeds of the other species were from the storehouse of the Horticulture Department of Bath University.

For most of the above-mentioned species there is tissue culture work done concerning the morphogenetic capacity of their tissues and potential for plant propagation (Bottino, 1980; Conger, 1980; Skirvin, 1980).

b-2. Isolation of the embryos

For dissecting the embryos of Pistacia terebintha the seeds were mixed with sand and they were rubbed repeatedly between two pieces of board until the fleshy green pericarp and mesocarp were removed. The remaining stones were rinsed with water and left to dry well. To soften the woody endocarp the dry and clean stones were scarified with concentrated sulfuric acid (specific gravity 1.84) for 15, 30, 75, 60, 90 and 120 min (Heit, 1967). The stones were placed in glass containers (beakers) and covered with the acid in a ratio of about one part seed to two parts acid. The mixture was stirred cautiously at intervals during the treatment to produce uniform results and to prevent the accumulation of dark material from the woody endocarp. At the end of the treatment period the acid was poured off and the stones were washed with sterile water to remove the acid as quickly as possible, reduce the temperature and avoid splattering. For the embryo isolation after the proper time treatment the scarified stones were cut by using a scalpel.

Another way of dissecting the embryos was to crack the stones by using a vice but the previous way was preferred because it ensured sterility.

For the dissection of the olive embryos similar ways were followed. The olive stones were scarified with concentrated sulfuric acid for 1, 2, 4, 6 and 8h or were cracked by using a vice and the embryos were dissected out of the surrounding endosperm.

Embryos from bean, pea, cabbage, lettuce, celery, wheat, barley, apple and lemon were dissected after soaking the seed in water overnight. Before maceration embryos from Prunus species were also soaked in water overnight and their testa was removed.

b-3. Maceration of embryos

Isolated embryos of the above species except celery were cut in small pieces of such a size so as to be able to enter a Pasteur pipette. The pieces of embryos were treated with 1% Macerozyme and 0.1% Rohament P for 2h at 32°C. After rinsing with 0.6M mannitol and postdigesting for 24h at 27°C they were sheared by using a 5ml syringe with a number V needle. The resulting cell suspension was poured through a 63µm mesh fixed in a Millipore filter holder to remove any debris and was made up to volume with 0.6M mannitol solution for the estimation of cell number.

Celery embryos were similarly treated but intact. For their shearing a 5ml syringe with a number VI needle was used and the cell suspension was poured through a double 45µm steel mesh.

With large embryos like bean and pea, embryo axes and cotyledons were also macerated separately.

c. Effect of lipases on the carrot embryo cuticle

The lipases [triacylglycerol lipase, triacylglycerol acyl hydrolase] supplied from Sigma (Sigma Chemical Company) tested for the digestion of the cuticle at the following concentrations per 15 embryos were: 1) No L-3001 from wheat germ, type I 100mg. 2) No 3126, type II, crude from hog pancreas, containing amylease and protease activity, 60mg. 3) No L-1754, type VII from Candida cylindracea, substantially free of α -amylase and protease, 2mg and 4) No L-1505, type IX from Rhizopus arrbizus. Suspension in 3.2M $(\text{NH}_4)_2 \text{SO}_4$ solution pH approximately 7, 0.004ml. All the solutions were 0.6M in respect to mannitol and their pH was 7.

A number of carrot embryos was treated as previously described, i.e., with 1% Macerozyme while other embryos were similarly treated with 0.1% Rohament P plus 0.1% Cellulase präp 223oA. After 24h postdigestion in 0.6M mannitol when the cuticle had been completely separated from the embryonic cells the embryos were transferred to the lipase solutions incubated at 32°C and observed after 1, 2, 3, 6, 12 and 24h. There were six replicates per treatment each one containing 15 embryos.

d. Embryo viability test

The 2, 3, 5 triphenyltetrazolium chloride (TTC) test (Lakon, 1949) was used for checking embryo viability. With this test, which distinguishes between living and dead tissues within a single seed, either seeds having 100% viable embryonic tissues

could be selected or the percentage of the viable embryonic tissues could be assessed. So after cell isolation any loss or further reduction in cell viability would be because of the macerating method.

The embryos or seeds were incubated overnight in a 1% TTC solution at room temperature in the dark.

In using the test certain procedures were followed. Any hard covering such as an endocarp was removed, seeds with fragile coverings like beans, peas and Citrus were softened slowly on moist absorbing paper to avoid fracturing and embryos with large cotyledons such as Prunus, apple, olive and Pistacia terebintha were excised completely for a better TTC absorption. With carrot and cereals a cut was made close to the end of the seed opposite to the one containing the embryo and after incubation the embryos were squeezed out (carrot) or dissected (cereals) and their viability was assessed.

e. Viability test for carrot embryos isolated and macerated with Macerozyme

Squeezing out carrot embryos is a time-consuming process and embryos had to remain in water for a considerably long time until the necessary number of them was obtained. To investigate whether squeezing out embryos is a process affecting the embryo viability and whether the stay of embryos in water or the post-digestion in 0.6M mannitol solution after maceration with 1% Macerozyme reduces the viability of embryonic tissues, isolated carrot embryos were treated with 1) 1% TTC solution as previously described and 2)

Nitro-blue-tetrazolium (Nitro-BT) (Hauser and Morrison, 1964).

Incubations with the Nitro-BT medium (see Appendix 1 for its composition) were carried out at 37°C for 45 min and the pH of the medium was adjusted to 7.2.

The treatments were given to the embryos after they had remained for 0, 24, 48 and 96h in water or been post-digested for the same times in 0.6M mannitol solution after the enzymic maceration. There were three replicates per treatment each with 20 embryos. During the experiment embryos were kept in 10ml glass vials covered with plastic tops and containing 2ml of each of the solutions involved i.e. water, 0.6M mannitol, TTC and Nitro-BT.

f. Cell viability

Cells isolated from embryos of the different species were tested for their viability. The dyes involved were 0.5% phenosafranine (Widholm, 1972) and 0.25% Evan's blue (Gaff and Okong O'-Ogloa, 1971). A drop of the cell suspension was mixed with a drop of 1% phenosafranine or 0.5% Evan's blue solution containing 0.6M mannitol so that the final concentration was 0.5% and 0.25% respectively. After 5 min the cells were observed with a microscope and the number of stained and not stained cells was counted. Viability was expressed as the percentage of the cells which retained their colour.

Protoplasmic streaming could not be observed because of the big organelles the embryonic cells had, and therefore it could not be used as a criterion for cell viability.

g. Statistical analysis

Statistical analysis of the data of this and the other chapters of this work was conducted using analysis of variance. In tables, numbers accompanied by different letters show that these numbers differ significantly. Numbers accompanied by the same letter do not show statistically significant differences. The least significant difference (LSD) for probability 95% is written below the tables.

C. RESULTS

a. Isolation of cells from carrot embryos

a-1. Morphology of zygotic carrot embryos and morphological aberrations

In this work thousands of carrot embryos were squeezed out of carrot seed and any aberrations from their regular shape were recorded. Zygotic embryos of the var. Chanteney Red Core consist of two well developed cotyledons and an axis bearing the shoot meristem on the one end between the two cotyledons and the radicle at the opposite end. The average embryo length was found to be 0.94mm, the average length of the radicle 0.56mm and the average diameter of the radicle 0.20mm. These numbers are the means of measurements made at 100 zygotic embryos isolated from randomly selected seed and they reflected a range of 0.57-1.34, 0.31-0.94, and 0.13-0.28mm for the embryo length, radicle length and diameter respectively.

It was observed that the size of the embryo could not be related to the size of the seed. Sometimes very small embryos came out of big seed and bigger embryos came out of smaller seed. In certain cases embryos were found to be folded within the seed and these embryos were longer than the seed itself.

The aberrations of the morphology of embryos recorded throughout this work concerned mainly the number of cotyledons per embryo. About 2% of the isolated embryos had three cotyledons instead of two, and in total only two embryos were found to have one cotyledon instead of two (Fig. 1a). Dimensions of embryos with three cotyledons were within the range mentioned previously for

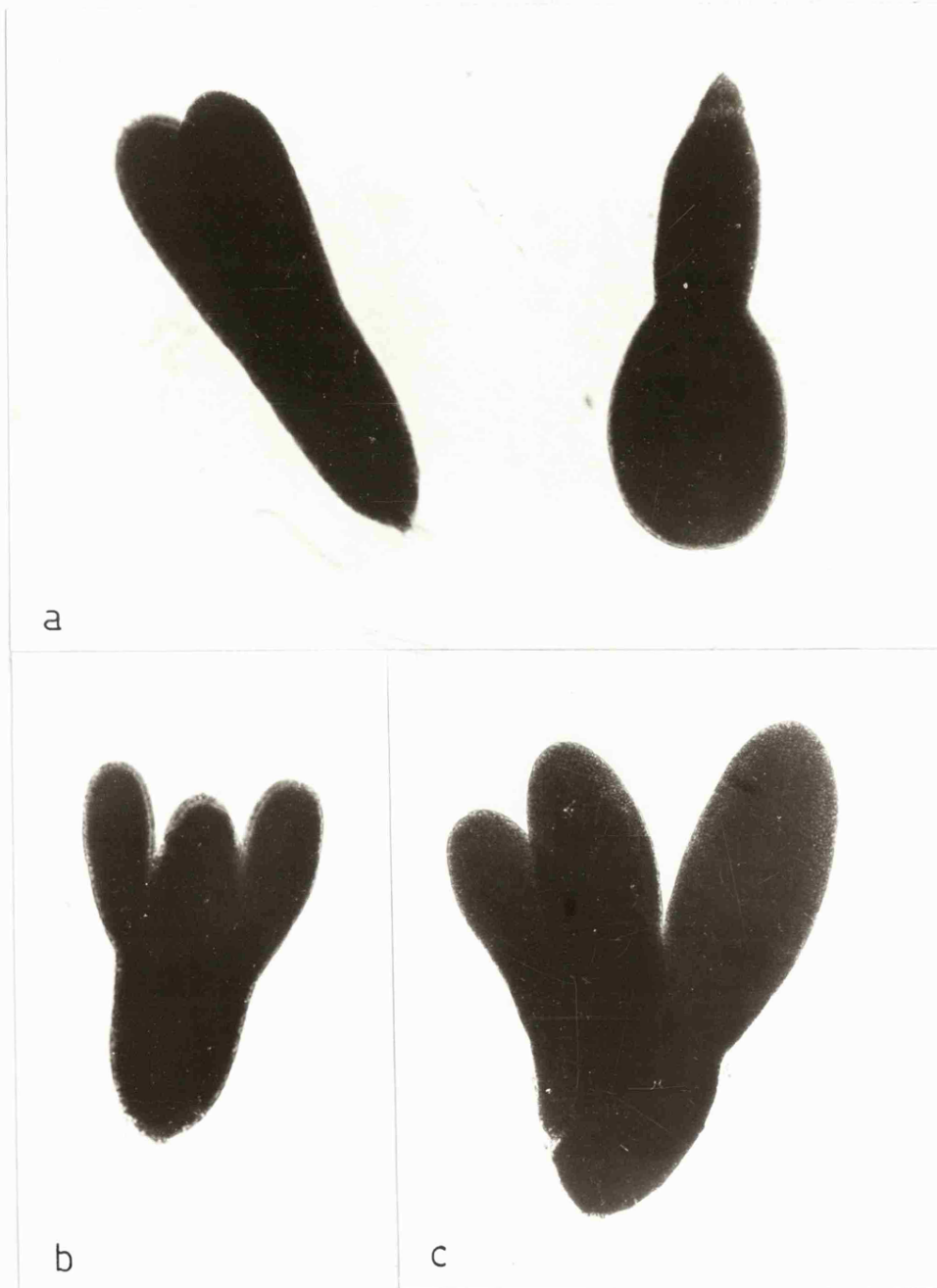


Fig. 1: a. A zygotic carrot embryo, var. Chanteney Red Core, bearing one cotyledon in comparison to an embryo having two cotyledons. b-c. Zygotic carrot embryos of the same variety bearing three cotyledons.

normal embryos, but sometimes the three cotyledons were overdeveloped in relation to the radicle which remained very small, almost as a root meristem from which big cotyledons arose (Fig. 1b-c).

a-2. Carrot embryo cuticle

Observation of post-digested carrot embryos under the microscope revealed the existence of a cuticle (Fig. 2). This cuticle is continuous, it covers the whole embryo and it is stained red by Sudan IV in saturated alcoholic solution for a quarter of an hour according to Johansen (1940). All the enzymes used in this work (see section Materials and Methods) separated the cuticle from the embryonic cells. This separation could be observed after enzymic digestion but it was complete after 24h post-digestion in 0.6M mannitol.

The cuticle was first observed before shearing of embryos with a hypodermic syringe was adopted in the method. It was thought that the cuticle surrounding the embryo was acting as an envelope not allowing the cells to be released. Experiments with macerated pieces of embryos which were not completely surrounded by the cuticle gave the same results suggesting that apart from the cuticle acting as an envelope a strong mechanical effect was also necessary for the release of the cells.

It was concluded that the carrot embryo cuticle was permeable to all the cell-separating enzymes used in this work and to other long molecules like TTC and Nitro-BT.

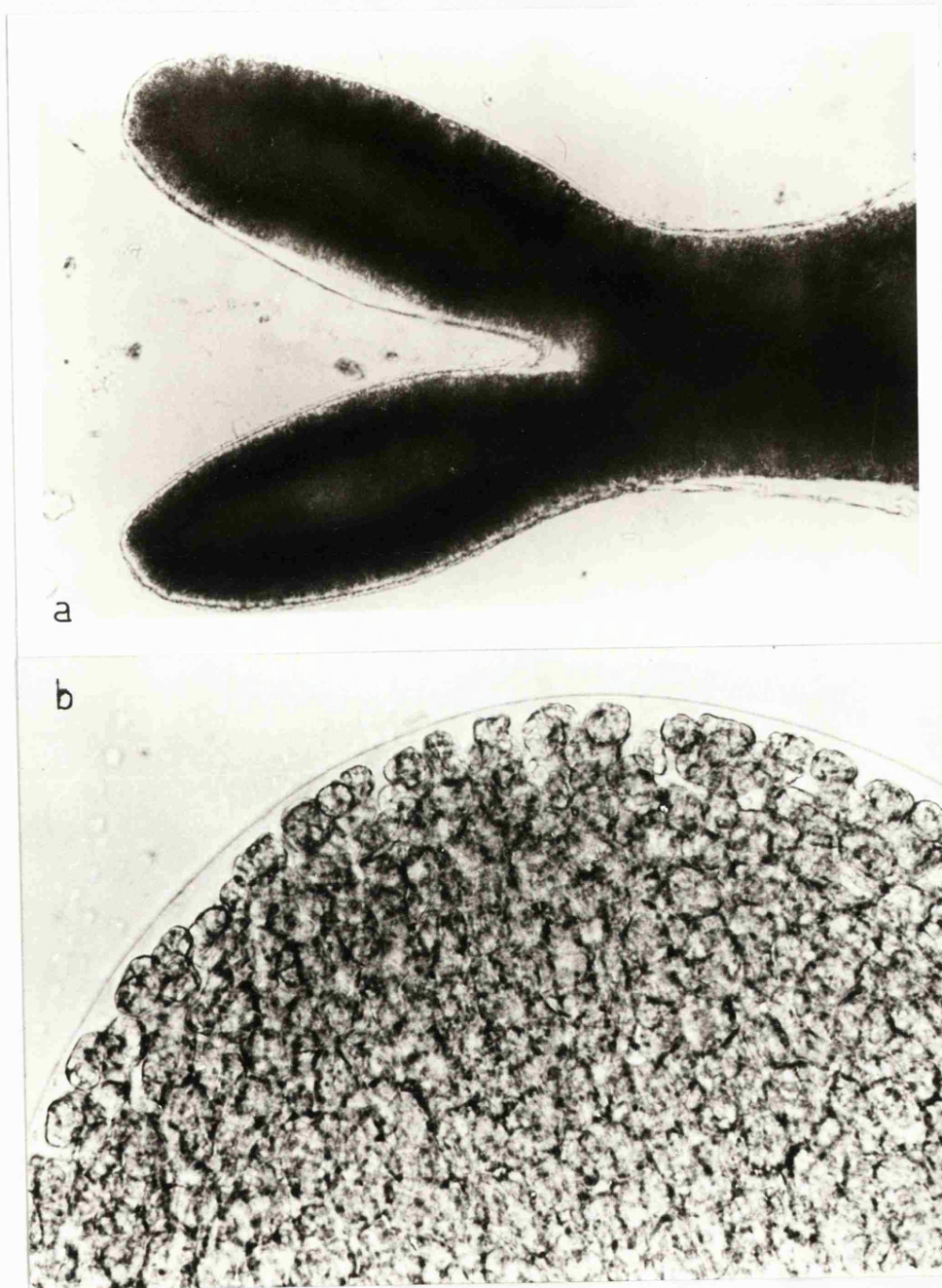


Fig. 2: Carrot embryo cuticle. Round the embryo (a) and on the outside of the cotyledon (b).

a-3. Effect of lipases on the carrot embryo cuticle

From the idea that cells could be released by simply agitating macerated embryos when the carrot embryo cuticle had possibly been removed or digested the effect of different lipases on the digestion of the cuticle was studied. Cuticles of carrot embryos digested with Macerozyme or with Rohament P plus Cellulase präp 223oA remained intact after the different lipase treatments. The enzymes permeated the cuticle and after an incubation of 24h some embryo cells were digested and the space they had occupied remained empty inside the cuticle.

a-4.1. Maceration of carrot embryos with Macerozyme

Digestion with 1% Macerozyme for 2h was enough to allow the enzyme to penetrate the embryonic tissues and 24h post-digestion in 0.6M mannitol completed the separation of cells. Macerated embryos passed through the needle of the syringe in spite of the fact that their dimensions were bigger than the internal diameter of the needle. This happened because maceration softened the embryonic tissues.

After shearing, 100% of the cells were released as single cells and the maximum number per embryo was in between 15×10^3 - 16×10^3 . Cells were morphologically intact except some, about 4%, which had partly digested walls (Fig. 3). This was due to the cellulase activity contained in the Macerozyme preparation. The average cell size was $14 \times 10 \mu\text{m}$ reflecting a range of 25-6 μm length and 18-5 μm width.

Immediately after shearing very few cells already under-

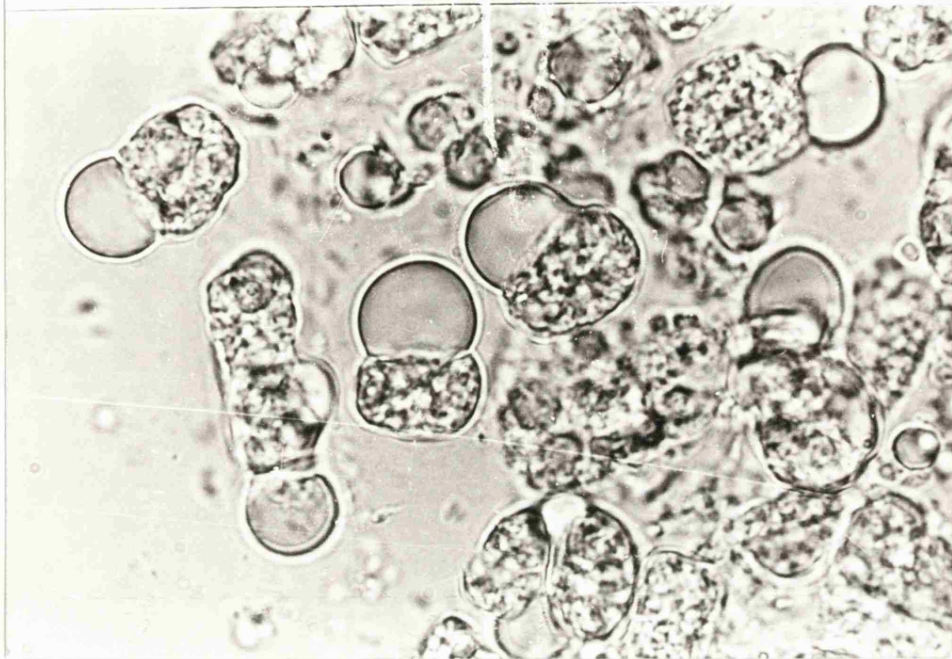


Fig. 3: Cells isolated from zygotic carrot embryos, var. Chanteney Red Core, showing cell wall digestion after treatment with 1% Macerozyme for 2h at 32°C and 24h post-digestion in 0.6M mannitol.

going division were observed. This indicated that while embryos were in post-digestion separated cells within the embryo were capable of division.

The viability of the released cells as tested with the use of Evan's blue was 90%.

a-4.2. Variation of Macerozyme concentration

Embryos were incubated with the different Macerozyme concentrations for 2h at 32°C and post-digested in 0.6M mannitol solution for 24h. In this experiment the effect of the Macerozyme concentration (0, 0.5, 1, 2 and 3% w/v, three replicates of 20 embryos per treatment) on the release of single cells from carrot embryos was assessed.

With 0% Macerozyme embryos could hardly pass through the needle of the syringe and those which did pass were broken into pieces. After shearing whole embryos and big cell aggregates could be observed but no single cells. With 0.5% Macerozyme cell aggregates consisting of 2-8 cells were in the cell suspension while with concentrations 1% and higher there were no cell aggregates. Concentrations of 2 and 3% though increased the number of cells having partly digested walls up to 15% in comparison to 4% with 1% Macerozyme. The number of cells released per embryo was approximately the same for 1, 2 and 3% concentration but lower with 0.5% (Table 1).

Table 1

Effect of the concentration of Macerozyme on the
release of single cells from embryos of *Daucus carota* L. var.
Chanteney Red Core.

Enzyme concentration % w/v	Number of cells ₃ per embryo x 10 ³
0.5	10.10 a
1.0	15.85 b
2.0	15.34 b
3.0	14.65 b

LSD (.05) = 1.63

a-4.3. Variation of the time of digestion with Macerozyme

To find the optimum time with respect to the number of cells released per embryo for the digestion of embryos with 1% Macerozyme at 32°C with 24h post-digestion in 0.6M mannitol, carrot embryos were digested for 0, $\frac{1}{2}$, 1 $\frac{1}{2}$, 2, 2 $\frac{1}{2}$ and 3h. There were three replicates per treatment, each one containing 20 embryos.

With no digestion time embryos could not pass through the syringe and there was no cell release. Half and 1h digestion times resulted in the release of a significant number of cells per embryo but cell groups consisting of 2-8 cells could also be observed. The highest number of cells was released with 1 $\frac{1}{2}$ and 2h digestion time. At these times separation of cells was complete and after shearing no cell groups were observed. With longer times of digestion the number of cells per embryo was diminished while the percentage of cells having their walls partly digested was up to 15%. The explanation for the decreased number of cells released per embryo with 2 $\frac{1}{2}$ and 3h (Table 2) is that cells having digested walls or cells which possibly were converted into protoplasts could not resist the fierce shearing and broke.

a-4.4. Variation of the digestion temperature

The temperatures chosen for this experiment were 27, 32, 37 and 42°C and embryos macerated with 1% Macerozyme for 2h were sheared after no post-digestion and after 24h post-digestion time. There were three replicates per treatment each containing 20 embryos. The aim of using the relatively high temperatures of 37 and 42°C was to possibly speed up the separation of cells and make it to be completed

Table 2

Effect of the time of digestion on the release of
single cells from embryos of *Daucus carota* L. var.
Chanteney Red Core.

Digestion time hours	Number of cells ₃ per embryo x 10 ³
$\frac{1}{2}$	9.23 a
1	12.98 b
$1\frac{1}{2}$	15.23 c
2	15.86 c
$2\frac{1}{2}$	13.75 bc
3	11.25 b

LSD (.05) = 1.96

within the 2h of enzymic maceration. Another reason was that the optimum temperature for Macerozyme is in between 40-50°C (Yacult Biochemicals Co. information bulletin, 1975). If separation of cells could occur with 2h digestion then any post-digestion in mannitol would be unnecessary.

With no post-digestion time the number of cells released per embryo was relatively low at 27°C (Table 3) while with the other temperatures this number was roughly the same. Cell separation was not complete since cell aggregates observed under the different temperature treatments the number of them being higher with lower temperature. With 37°C and 42°C apart from the cell aggregates, cells having their walls partly digested could be observed. This indicated that while in certain areas of the embryo the cell separation was still in process, in others it had already been completed.

With 24h post-digestion time the maximum number of cells per embryo was obtained at 32°C. At this digestion temperature there were no cell aggregates, and cells distorted because of cellulase activity were approximately 4%. With 27°C the number of cells per embryo was lower obviously because cells were not completely separated and a few cell aggregates consisting of 2-4 cells could be observed. With 37°C and 42°C cell number per embryo was also lower than with 32°C. There were no cell aggregates but there were up to 20% cells having partly digested walls (Table 3B). With these temperatures the lower number of cells is explained by the fact that cells with digested walls broke during shearing.

Table 3

Effect of digestion temperature in combination with no and 24h post-digestion time on the release of single cells from embryos of *Daucus carota* var. Chanteney Red Core.

Temperature	Total number of cells x 10 ³		Single, morphologically intact and viable cells x 10 ³	
	No post- digestion	24h post- digestion	No post- digestion	24h post- digestion
27	9.98	10.57	8.83	7.93
32	15.31	16.53	13.47	14.21
37	15.98	13.85	13.42	11.22
42	14.99	12.21	11.69	8.54

Table 3B

The effect of temperature and post-digestion time on the number of cells having partly digested walls with 1% macerozyme and 2h digestion.

Temperature C°	Percentage of cells	
	with no post-digestion	with 24h post-digestion
27	0.5	1.5
32	2	4
37	6	9
42	12	20

a-4.5. Variation of post-digestion time

Because maceration of embryos at a range of Macerozyme concentrations, digestion times and temperatures did not result in 100% single cell release embryos were post-digested in 0.6M mannitol for 0, 12, 24, 48 and 96h (three replicates per treatment each containing 20 embryos). Although the embryos were rinsed after enzymic digestion, cell separating processes continued during post-digestion because the enzyme penetrated the embryonic tissues and it can be seen from the data (Table 4) that they were completed after 24h post-digestion in mannitol. With this time the highest number of cells per embryo was released, no cell aggregates were observed and the percentage of cells having digested walls was approximately 4%. With 0 and 12h digestion the number of cells released per embryo was smaller and cell aggregates could be observed. The number of cells released per embryo was also smaller with 48 and 96h post-digestion. This is explained by the fact that many cells burst during shearing. Of the counted cells 40% and 100% had partly digested walls after 48 and 96h respectively. Very few protoplasts also which surprisingly survived shearing were observed after these times of post-digestion.

The same experiment was repeated with post-digesting the embryos for the same times in the basic media of Murashige and Skoog (1962), Fujimura and Komamine (1975) and White (1943) containing no growth regulators and 3%, 2% and 2% sucrose respectively. After 12h embryos were sheared with difficulty and cells were released mainly as cell aggregates. After 24h the embryos could not be sheared and the needle of the syringe was blocked. Obviously the

Table 4

Effect of post-digestion time on the release of
single cells from embryos of *Daucus carota* L. var.

Chanteney Red Core.

Post-digestion time hours	Number of cells ₃ per embryo x 10 ³
0	10.18 a
12	13.56 b
24	16.85 c
48	13.74 b
96	7.56 d

LSD (0.5) = 0.98

connections among the cells had already been re-established. This not only could not happen while embryos were starving in 0.6M mannitol but on the contrary cell separation processes continued and were completed after 24h.

a-4.6. Maceration of carrot embryos with other cell-separating enzymes

This experiment was a comparison of different commercial enzymic preparations on the release of single, morphologically intact and viable cells from carrot embryos. The results obtained with these enzymes were compared with those obtained with Macerozyme (Table 5). There were three replicates per treatment each one containing 20 embryos.

Maceration of carrot embryos with Macerase was tried at 0.5, 1, 2 and 3% w/v concentrations. Best results obtained at the concentration of 1%. 100% of the cells were released, no cell aggregates could be observed and cells having partly digested their walls were about 6%. The viability was 80%. With lower than 1% concentrations the number of cells released per embryo was smaller and cell aggregates could be observed. With higher concentrations the cell number per embryo was approximately the same but about 18% of the cells had digested walls because of the cellulase activity contained in Macerase.

With Rohament P embryos were macerated at concentrations of 0.01, 0.05, 0.1 and 1% w/v. The highest number of cells released per embryo was with 0.1% and higher concentrations. The main characteristic of the cells released was that there was too little

Table 5

Effect of various cell-separating enzymes on the release of single, viable and morphologically

intact cells from embryos of *Daucus carota* L. var. Chanteney Red Core.

Enzyme	Number of cells per embryo x 10 ³	Viability %	Viable cells per embryo x 10 ³	Cells having digested walls %	Morphologically intact cells per embryo x 10 ³	Morphologically intact and viable cells per embryo x 10 ³
Macerozyme	15.85	90	14.26	4	15.21	13.69
Macerase	15.18	80	12.14	6	14.27	11.41
Rohament P	14.13	70	9.89	0.5	14.06	9.84
Rohament P + Cellulase Präp 223oA	15.21	55	8.36	12	13.38	7.36
Pectinase SS	15.67	70	10.97	10	14.10	9.87

cellulase activity. Cell viability was 70%. With lower than 0.1% concentrations maceration of embryos was not complete, the number of cells released per embryo was smaller and cell aggregates were observed. When the same Rohament P concentrations in combination with 0.1% Cellulase präp 223o A were used the number of cells released per embryo was increased under all treatments but the cell viability was reduced to 55% and with 0.1% and higher concentrations about 12% of the cells showed cellulase activity.

Pectinase SS was effective at higher concentrations in comparison to the previous enzymes. From the concentrations 0.5, 1, 2 and 4% w/v tested in this experiment best results were obtained with 2%. Cell viability was 70% and cells having digested walls were 10%. With lower than 2% concentrations cell aggregates were observed while with higher 20% of the cells showed cellulase activity.

a-5. Maceration of "germinating" carrot embryos

Carrot seeds were put on a moist absorbing paper in a Petri dish kept at room temperature in order to induce germination. Carrot embryos were isolated at two different stages, first when the emerging root was 2mm long and second when the emerging root was 4mm long. The control in this experiment was embryos isolated from seed presoaked in water. Maceration was with 1% Macerozyme for 2h at 32°C and 24h post-digestion in 0.6M mannitol solution.

While embryos isolated from seed presoaked in water were completely macerated after shearing and 100% of their cells were released as single cells, there was too little, almost no, cell release from embryos isolated at two different germination stages.

Either the embryos were macerated intact or cut in small pieces.

a-6. Carrot embryo viability test

When the TTC test was applied directly to carrot embryos it did not work. Embryos incubated in Nitro-BT incubation medium became blue. If the Nitro-BT was replaced by TTC at ten times higher concentration the embryos after incubation turned to red colour. Sodium succinate was the necessary substrate for the reaction of the TTC in the carrot embryo tissue. When seeds were tested only with the TTC the endosperm provided the embryos with succinate, and if the embryos were viable they were stained red.

b. Isolation of cells from embryos of other species

b-1. Isolation of embryos

With Pistacia terebintha seed best results were obtained with 60 min acid scarification. At this time the woody endocarp had become paper thin and it could be cut easily using a scalpel in order to isolate the embryo. Longer times resulted in over-digestion of the endocarp and the acid came in contact with the embryo and killed it. With olive stones acid scarification did not give good results even after 8h. The endocarp which had a thickness varying from 1-2mm did not become thin enough to cut easily.

Cracking the stones using a vice proved to be a rough way for isolating Pistacia terebintha embryos since crushing of embryos was unavoidable. On the contrary for olive stones this way was adopted firstly because the acid treatment did not give the expected results for the times used and secondly because olive seed could be sterilized without any damage to the embryo due to the sterilizing agent. The olive embryo is surrounded by the relatively hard endosperm and the testa outside the endosperm. Embryos were isolated after cutting the seed longitudinally.

It was easy to isolate embryos from presoaked seed of other species, just by removing the softened testa and possibly the endosperm. Celery embryos were isolated in the same way as carrot embryos, i.e., by squeezing the presoaked seed.

b-2. Embryo development at maceration time

From the dicotyledonous species used in this work olive and celery and carrot had endospermous seed while all the others had non-endospermous with massive food storing cotyledons (Fig. 4). Embryos from species with non-endospermous seed showed a variation on their development. For example the apple embryo had a very small embryo axis and any leaves apart from cotyledons could hardly be seen on isolated embryo axes by visual observation. Bean and pea embryos had a very well-developed embryo axis with distinct root and leaves, being like a little plant, between the two big cotyledons. All the other dicotyledonous species had embryos at an intermediate stage of development in comparison to apple and bean or pea.

Embryos of Pistacia terebintha although smaller in size were at a similar stage of development in comparison with bean embryos. Their axes had a well-developed root and two small but fully differentiated leaves. The very interesting thing with Pistacia terebintha embryos though was that all the embryos isolated in this work were chlorophyllous. The green colour was darker in cotyledons and lighter along the embryo axis. It was surprising that chlorophyll could be formed in Pistacia terebintha embryos which had never been exposed to light before and were serially surrounded by the woody endocarp and the fleshy mesocarp and pericarp.

Embryos from endospermous seeds did not show any variation in the development of their embryos, all of them being at the cotyledonary stage. The only exception was with olive. It was observed only twice throughout the whole series of experiments that the embryo was in an advanced stage of development having two leaves

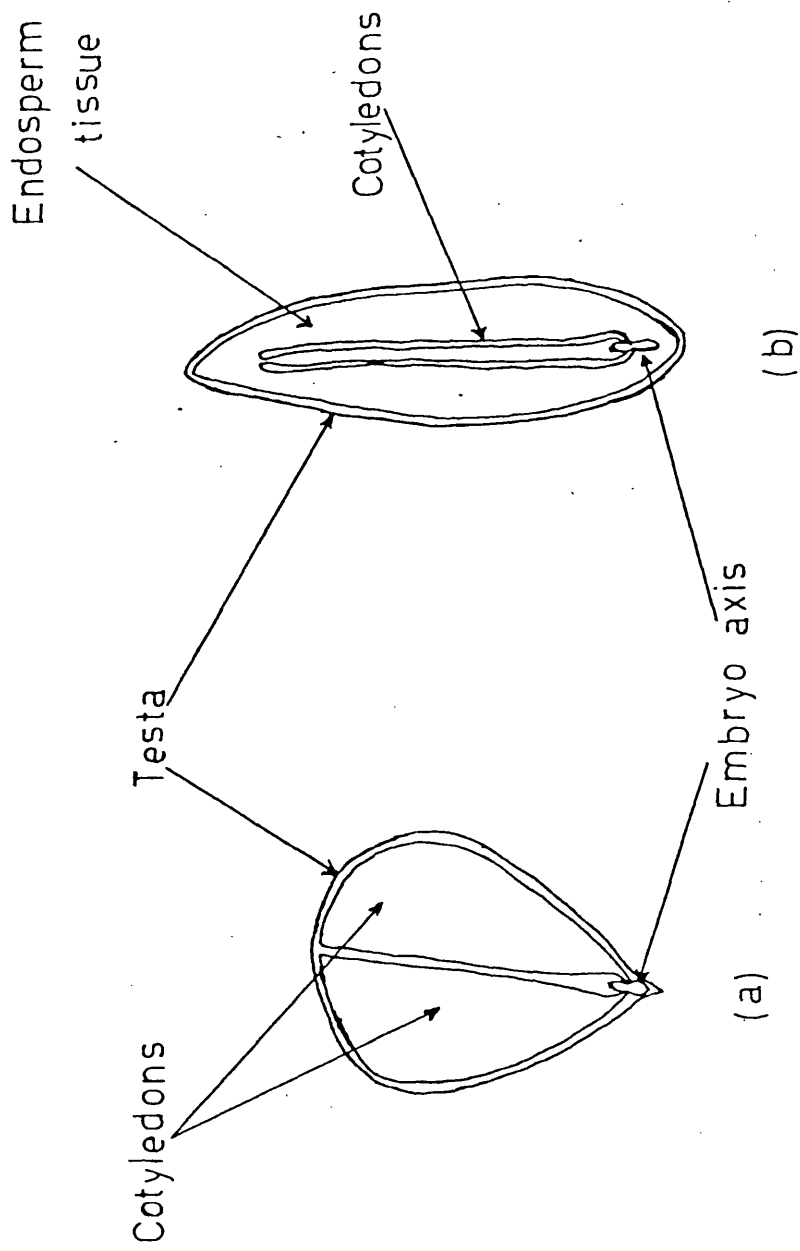


Fig.4 : Seed structure of (a) apple (non endospermous) (b) olive (endospermous) without the woody endocarp

3mm long each and an embryo axis 3mm long and 1.5mm thick. There were no cotyledons and the embryo was a little plantlet surrounded by the half spent endosperm. Obviously the cotyledons and part of the endosperm were spent for the development of the embryo which later for some reasons was inhibited.

With the monocotyledonous species, barley and wheat there was no variation of the embryo development.

b-3. Aberrations of embryo morphology

As with carrot, apple embryos having three cotyledons were observed as well. The frequency of the three cotyledon embryos was higher, up to 5% with certain lots of apple seed of the cv. Golden Delicious but lower 0.2% with seed of the cv. Phiriki.

No morphological aberrations were observed with embryos of the other species.

b-4. Embryo viability

After seeds were treated with 1% TTC viable embryonic tissues were stained red while dead retained their original colour. The embryos from all the species except almond were completely coloured. This indicated that 100% of the embryonic tissues was viable. With almond embryos there were unstained spots depending upon the amount and location of necrotic areas. It was estimated that approximately 60% of the embryonic tissues was viable (stained) and the remaining 40% dead (unstained).

b-5. Maceration of embryos and cell isolation

The number of cells released per embryo of some of the various species used in this work, the percentage of cells having partly digested cell walls, and their viability are shown in Table 6. This number is higher with 1% Macerozyme and lower with 0.1% Rohament P. Fig. 5 shows single cells isolated from various species. Apple, Pistacia terebintha, bean and pea embryos were completely macerated and 100% of their cells could be released as single cells even immediately after enzymic digestion with no post-digestion time in 0.6M mannitol solution. For bean and pea embryonic tissues (including the fully differentiated young leaves) it was enough to expel them only once through the syringe to release their cells. Obviously the enzyme penetrated these tissues easily and quickly, the cell separation was completed within the 2h enzymic digestion and a light mechanical effect was enough for the cells to be released. For celery embryos 24h post-digestion in mannitol was necessary. Unlike the other tissues, embryos, fragments and cells of Pistacia terebintha floated on water and mannitol solution. Tissues of Pistacia species are known to contain resinous substances (Sficas, 1978). Pistacia terebintha embryonic cells probably have a high content of such substances which are lighter than the water and float.

Maceration of wheat, barley and olive embryos proved to be difficult and only part of their tissues was released as cells. The reason for that seemed to be a difficulty of the enzymes to penetrate the tissue and separate the cells. Experiments which included the involvement of wetting agents like Tween 20 in com-

Table 6

Number of single, viable and morphologically intact cells released per embryo of various species by using 1Z Nucerozyme and 0.1Z Rohment P.

Enzyme	Plant species	Number of cells, per embryo $\times 10^3$	Viability %	Viable cells per embryo $\times 10^3$	Cells having digested walls %	Morphologically intact cells per embryo $\times 10^3$	Morphologically intact and viable cells per embryo $\times 10^3$
1Z Nucerozyme	Brassica oleracea var. Coleslaw	349.54	100	349.54	0.5	347.80	347.80
	Hordeum vulgare var. Maris Otter	114.54	70	80.18	6	107.68	75.37
	Malus domestica var. Phiriki	1,784.35	90	1,605.92	2	1,748.66	1,573.80
	Olea europaea var. Amphissis	989.76	90	881.79	5	940.28	837.70
	Phaseolus vulgaris var. Canadian Wonder	10,170.25	40	4,068.10	4	9,763.45	3,905.38
	Pistacia terebintha	1,090.65	95	1,036.12	3	1,057.84	1,005.04
	Prunus mahaleb	2,875.98	75	2,156.99	2	2,818.46	2,113.85

Table 6 (continued)

Number of single, viable and morphologically intact cells released per embryo of various species by using 1% Macerozyme and 0.1% Rohament P.

Enzyme	Plant species	Number of cells per embryo x 10 ³	Viability %	Viable cells per embryo x 10 ³	Cells having digested walls %	Morphologically intact cells per embryo x 10 ³	Morphologically intact and viable cells per embryo x 10 ³
0.1% Rohament P	Brassica oleracea var. Coleslaw	334.15	85	284.03	0	334.15	284.03
	Hordeum vulgare var. Maris Otter	25.97	65	16.88	1	25.71	16.51
	Malus domestica var. Phiriki	1,301.26	80	1,041.01	0	1,301.26	1,041.01
	Olea europaea var. Amphissis	415.89	80	332.71	1	411.73	329.38
	Phaseolus vulgaris var. Canadian Wonder	9,587.96	30	2,876.39	0.5	9,540.02	2,862.01
	Pistacia terebintha	1,081.34	70	756.94	0.5	1,075.94	753.16
	Prunus mahaleb	1,099.36	55	604.65	0	1,099.36	604.65

This experiment was carried out with a different batch of Macerozyme. 7% of the cells isolated from carrot embryos by using 1% Macerozyme of the same batch were having partly digested cell walls.

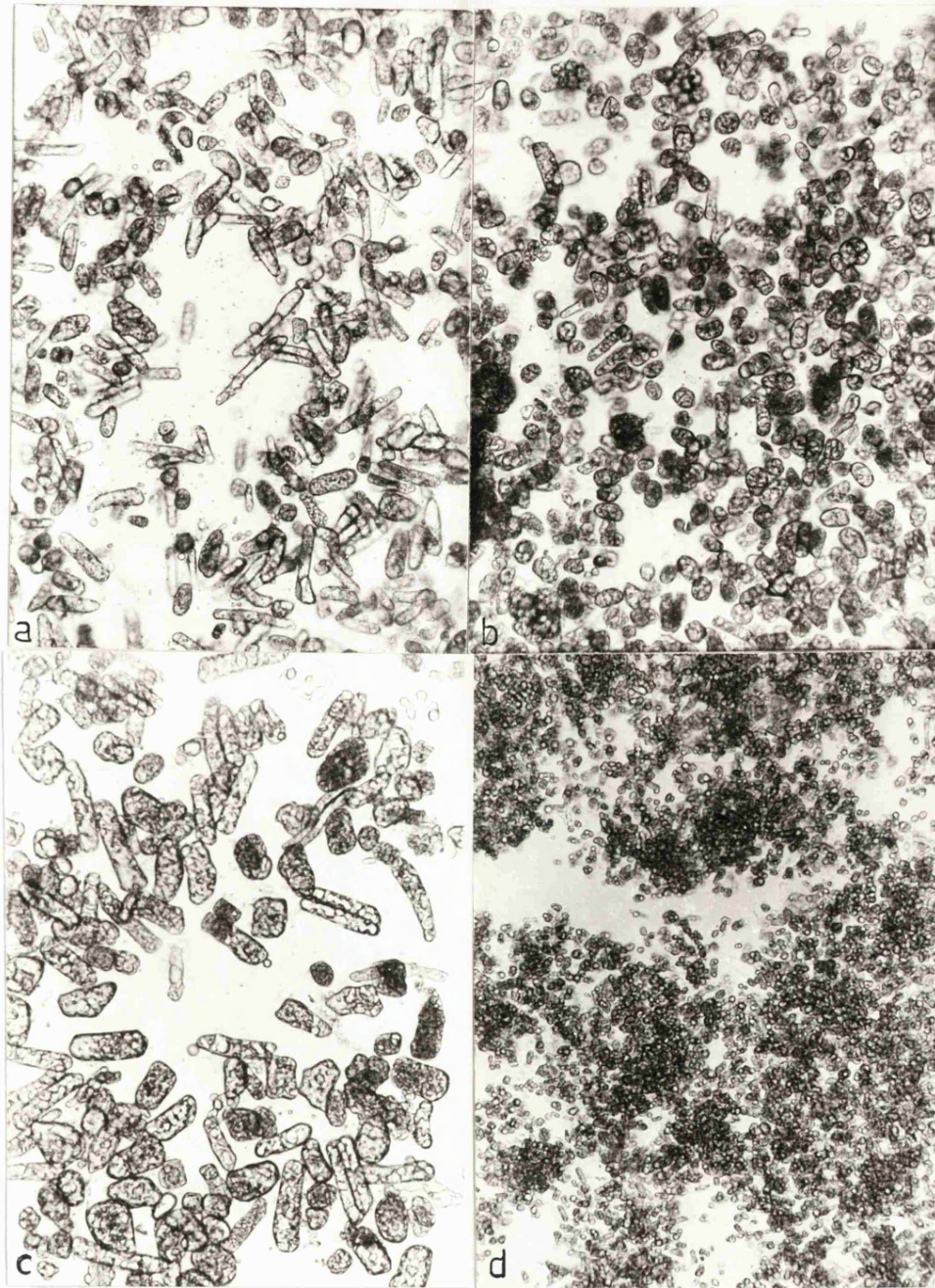


Fig. 5: Single cells isolated from zygotic embryos of apple (a), cabbage (b), lettuce (c) and carrot (d). a, b x 110, c x 150, d x 100.

ination with different enzyme treatments, overnight enzymic digestions with low enzyme concentration, i.e. 0.5% w/v Macerozyme, starvation in 0.6M mannitol for various times and preplasmolyzing the embryonic tissues in 0.8M mannitol for 6h did not show any further increase of the number of cells released per embryo. After all the above treatments and after shearing from the pre-digested pieces of embryos a relatively small number of cells was released while the bulk of each piece remained unmacerated. It was suggested that the cells released were from the superficial part of the pieces which came in direct contact with the enzyme.

The physiological status of the olive embryo seemed to have a positive effect on the release of single cells. Olive embryos are dormant and germinate after they have been stratified at temperatures lower than 20°C, the optimum being 13°C. A separate experiment was set up to compare the number of cells released from dormant and after-ripened olive embryos. Isolated embryos were put in Petri dishes on wet absorbant paper and transferred in a dark incubator at 13°C. After 15 days for *Amphissis* and 20 days for *Chondrolia Chalkidikis* (when seeds started germinating) the experiment was set using 1% Macerozyme. "Germinated" embryos were discarded and only after-ripened but not "germinated" embryos were macerated. Cell number per embryo was increased with after-ripened embryos by a factor of approximately 5 (Table 7).

The cellulase effect (due to cellulases contained in the enzyme preparations used in this work) varied from species to species with the different enzymes treatment. In comparison with carrot the relevant sensitivity to the cellulase activity in a diminishing

Table 7

Number of single, viable and morphologically intact cells isolated from dormant and after-ripened embryos of two varieties of *Olea europaea*.

Variety	Number of cells released from ₃ dormant embryos x 10 ³	Number of cells released from after-ripened embryos x 10 ³
Amphissis	910.75	4,398.21
Chondrolia Chalkidikis	789.65	4,021.32

order is carrot, celery/barley, wheat/bean, pea/olive/lemon/
Pistacia terebintha/apple, Prunus species/cabbage/lettuce. Cells from cotyledons of bean and pea embryos were more sensitive to the cellulase activity than the cells from the embryo axis. Cells from cotyledons were converted into protoplasts if starved in 0.6M mannitol for 48h. Cells from embryo axes showed cellulase activity but very little in comparison to the cotyledonary cells. The different response of cells from a single embryo and from embryos of various species to the cellulase activity obviously reflects differences in the chemical composition of their walls.

Cell viability varied from one species to the other under the same enzyme treatment and with the same species under the different enzyme treatment. The highest viability 100% was observed with lettuce, Pistacia terebintha and cabbage embryo cells treated with Macerozyme while the lowest 30% with cells of bean and pea embryo axes treated with Rohament P.

b-6. Embryo cuticle

Observation of whole embryos of the other species macerated and post-digested in 0.6M mannitol revealed the existence of a cuticle. As with carrot embryo cuticle, these cuticles were continuous, surrounded the whole embryo and were separated from the embryonic cells by the Macerozyme, Macerase and Rohament P.

Cuticles could be observed easier along the periphery of the root tip, and phase contrast microscopy in some cases contributed to a better observation of them.

DISCUSSION

During embryo isolation from carrot seed it was observed that the embryo size could not be related to the size of the seed. This is confirmed by the work of Megerdichev (1974) who found that the size of a carrot embryo depends on the position of the seed in the inflorescence. According to his work, mean embryo size decreased from the periphery to the centre of the umbel.

Aberrations from the normal morphology of plant embryos are known to occur frequently in tissue and cell cultures. From this work in which a high number of zygotic carrot and apple embryos was isolated it was proved that morphological aberrations occurring with somatic embryos should not be attributed to the tissue culture conditions or they should not be attributed exclusively to them. Although no morphological aberrations were observed with embryos of other species than carrot and apple used in this work it is expected that they do occur. The fact that no aberration was observed with these species was probably because of the very low frequency they occur and because of the small number of isolated zygotic embryos.

These experiments revealed the existence of a zygotic embryo cuticle, which seems to have its origin at an early stage of the embryo development. Cuticles are known to exist in apices, primordia and leaves, petioles, stems and culms, fruits and seeds, sepals, petals and other floral parts and roots. They have an important role as a structural element, holding the cellular tissues compact and firm and above all, they hold an important position as the boundary layer between the body of the plant and its environment.

They are also involved in phenomena at the plant surface which according to Martin and Juniper (1970) are classified in three broad categories: physiological functions, interactions with chemicals and interaction with pathogens. The role of the cuticle as a structural element surrounding the aerial parts and the root and its involvement in phenomena at the plant surface speak about its biological importance. But what could the biological importance of a cuticle surrounding the zygotic embryo be? Esau K.(personal communication) commented that while still within the seed the embryo acquires the mechanism for developing a cuticle which it will need upon germination. Other questions are 1) When does this cuticle originate? Does it have its origin at the zygote or at a later stage of the embryo development? 2) How does this embryo cuticle compare with the cuticle surrounding the different organs like leaves? What are their functional differences and differences in chemical composition?

Shilde Rentschler in 1973 reported that during preparation of tobacco mesophyll protoplasts stripping of the lower epidermis could be replaced by 30-60 min incubation with Rohament P. This information in relation to the observation of the embryo cuticle, gave the idea to use Rohament P and the Pectinase SS for the maceration of embryos into single cells.

All the pectinases used in this work separated the cuticle from the epidermal cells but none of them caused digestion of the cuticle. Between the layer of the epidermal cells and the cuticle above there is believed in many plants to be a layer of pectin, which is believed to be continuous with the middle lamella of the

anticlinal walls of the epidermal cells (Martin and Juniper, 1970). This view which cannot be positively proved by either the existence of a ruthenium red positive band or by its isotropy (Roelofsen, 1959) is supported by the fact that pectinases in this work separated the cuticle from the epidermal cells. These results are in agreement with the results obtained by Orgell in 1955 who worked on isolating cuticles from leaves. Orgell, apart from supporting the view that between the epidermal cells and the cuticle above there is a pectin layer, found that the time necessary for the separation of the cuticle of leaves varies from one species to another (3-12hr for leaves of Convolvulus arvensis, Peperomia spp, Philodendron spp, rapid detachment for leaves of Phaseolus vulgaris and from 12h up to 3 days for leaves of Citrus spp, Nicotiana spp and Prunus spp, while the cuticles of Vitis spp, Ficus elastica and Zea mays separate very slowly or fail to separate at all). In this work 2h digestion with 1% Macerozyme at 32°C and 24h postdigestion in 0.6M mannitol separated the embryonic cuticles of all the species used.

The criteria for cell isolation were: 1) the single cell nature of the obtained cell suspension; 2) morphological intactness of the isolated cells, and 3) viability. Under the various Macerozyme treatments the highest number of single, morphologically intact and viable cells was obtained with 1% Macerozyme for 2h at 32°C and 24h post-digestion in 0.6M mannitol solution. Any deviation from these conditions resulted in incomplete maceration, the existence of cell aggregates and in the increase of the number of cells having partly digested walls. Viability was approximately 90% and varied

little with the different treatments.

Among the different enzymes tested on the isolation of single cells from carrot embryos, Rohament P either alone or in combination with Cellulase präp 223oA and Pectinase SS which are commercial preparations for the manufacture of fruit and vegetable juices yielded lower (but satisfactory for their grade of quality) number of morphologically intact and viable cells per embryo in comparison to Macerozyme and Macerase which are prepared as cell-separating enzymes. From the data of Table 5 it is concluded that Rohament P which showed the least cellulase activity in comparison with the other enzymes could be a very good cell-separating enzyme if higher cell viability could be obtained with it.

In order to assess embryo viability the TTC test was used over the germination tests for the following reasons. 1) Many seeds do germinate in spite of the fact that a big portion of the embryonic tissue is dead, and other seeds do not germinate because only the root meristem or only the root and shoot meristems are dead. With the method employed in this work viable and dead embryonic tissues are equally well macerated into single cells. By using the TTC test seed having 100% viable embryonic tissue could be selected and after enzymic maceration and shearing any reduction in cell viability was because of the macerating method. 2) The TTC test works regardless of the physiological conditions (i.e. dormancy) in the seed and 3) it is quick and simple.

By using the TTC test and the test with the Nitro-BT incubation medium it was found that carrot embryos retained their

viability after enzymic maceration and post-digestion in 0.6M mannitol. So the loss in cell viability of carrot embryo cells should be attributed only to the shearing effect which is a strong mechanical shock to the embryonic tissue.

Maceration of carrot embryos at different stages of germination did not have the same results. Macerozyme was effective in isolating single cells from carrot embryos only when embryos were isolated from seed pre-soaked in water. This obviously reflects differences in the chemical composition of the cells occurring during germination. The same explanation is given for the maceration of olive embryos which after after-ripening yielded an approximately five times higher number of cells. It is an indication that the after-ripening process and the switch on of germination are accompanied by changes in the chemical composition of cells.

Cell-separating enzymes are in fact complex mixtures of enzymes (Evans and Cocking, 1977). In this work it was shown in combination with Cellulase präp 223oA and Pectinase SS which are commercial preparations for the manufacture of fruit and vegetable juices yielded a lower (but satisfactory for their grade of quality) number of morphologically intact and viable cells per embryo in comparison to Macerozyme and Macerase which are prepared as cell-separating enzymes. From the data of Table 5 it is concluded that Rohament P which showed the least cellulase activity in comparison with the other enzymes could be a very good cell-separating enzyme if higher cell viability could be obtained with it.

Cell-separating enzymes are in fact complex mixtures of enzymes (Evans and Cocking, 1977). In this work it was shown that the cell-separating enzymes contained cellulase. The fact that different species showed different sensitivity to the cellulase effect of the same cell-separating enzyme indicates differences in the chemical composition of embryo cells of various species. Similar differences seem to exist with cells of different parts of the same embryo. With Phaseolus vulgaris embryos, for example, cells from cotyledons were more sensitive to the cellulase effect of Macerozyme in comparison to cells from the embryo axis.

From the various species tested for embryo cell isolation some like bean, pea, Pistacia terebintha and apple were very easily macerated into single cells and 100% of their cells was released as single cells while wheat, barley and olive were macerated with difficulty and only a low percentage of their cells was released. Maceration of embryos with pectinases is based on the digestion of pectin existing in the intracellular space. Orgell (1955) working on the basis that between the cuticle and the epidermal layer there is a pectin layer, could isolate cuticles from leaves of various species using pectinases. As mentioned earlier the time for the separation of the cuticle varied from one species to another. The results obtained in this work and those obtained by Orgell can be correlated and it is obvious that they are in agreement. For example in Orgell's work the cuticle of Phaseolus vulgaris leaves was rapidly detached, and in this work Phaseolus vulgaris embryos were the easiest to be macerated into single cells.

Embryo axes of bean and pea embryos are little plantlets

having fully differentiated leaves. Because they were very easily macerated into single cells it would be interesting to study their maceration into single cells at different stages of their development. Isolation of a high number of cells from a seedling would facilitate the establishment of cloned cell suspension cultures.

Apart from the existence of an embryo cuticle another interesting feature was the occurrence of chlorophyll in Pistacia terebintha embryos. Raptopoulos (1970) reported that the edible part (i.e. the embryo) of Pistacia vera seeds is green. It seems that the existence of chlorophyll in embryos is a characteristic of the family of Anacardiaceae to which Pistacia species belong. Yakovlev and Zhukova (1980) in their review "chlorophyll in angiosperm seeds" reported other six species of the family of Anacardiaceae which have chlorophyllous embryos.

The number of cells released per carrot embryo was approximately 15×10^3 . Warren and Fowler (1978) estimated that the cell number per an adventive embryo at young torpedo stage is in between $2 \times 10^3 - 3 \times 10^3$. The size of the embryos at the same stage is $0.3 \times 0.5\text{mm}$ but there is no reference to the cell size. These adventive embryos are bigger in comparison to the zygotic embryos of the var. Chanteney Red Core at the cotyledonary stage (0.2mm width \times 0.6mm length). Halperin (1966) found that zygotic carrot embryos, although they are in an advanced stage of development, are smaller than the adventive embryos of an earlier stage. He also found that zygotic embryos are smaller than the adventive, if the comparison is made when zygotic and adventive embryos have equal size of cotyledons. From the photos he quotes it seems that the

adventive embryo cell size is larger than the zygotic embryo cell size. The ratio $\frac{\text{adventive embryo cell size}}{\text{zygotic embryo cell size}}$ is higher than the ratio $\frac{\text{adventive embryo size}}{\text{zygotic embryo size}}$. This explains why zygotic embryos of the var. Chanteney Red Core have a higher number of cells than the adventive embryos Warren and Fowler worked with.

Table 6 shows the number of cells released per embryo of various species. This number is lower than the real number of cells per embryo because through the procedure for cell isolation there is a certain loss of cells. It is concluded from this work that embryos of the same species, when within the seed and before germination starts, are in the same developmental stage. Perhaps it would be interesting to find out if in spite of the differences in size among embryos of the same species, the cell number per embryo is a constant for each species or variety.

CHAPTER 3

CARROT EMBRYO CELL AND CARROT EMBRYO CULTURE

A. INTRODUCTION

After having developed a method for isolating embryonic cells, naturally the next step was to study the culturability of these cells. Among the various plant species of which embryonic cells were isolated carrot was first selected for studying the growth responses of its embryonic cells in culture. The reasons for selecting carrot were these. 1) Carrot cultures are known to be responsive to growth and morphogenesis. 2) There is a voluminous literature concerning a detailed research on growth and morphogenesis of tissue and cell cultures of carrot. 3) Primary embryonic cell cultures of carrot in case of a positive morphogenetic response might constitute a model for primary embryonic cell cultures of other species.

The cultures were established with a two-fold purpose. This was firstly to induce cell division and colony formation from single carrot embryo cells and secondly to see how single carrot embryo cells would respond when they were cultured in media which were already well established as media allowing embryogenesis with secondary tissue and cell cultures of carrot. From these media the Fujimura and Komamine (1975) medium (Appendix 2) adjusted to pH 5.4-5.5 before autoclaving was selected.

The main difficulty in this work was the isolation of carrot embryos under aseptic conditions which was tedious and time-consuming because of the small size of carrot embryo. The small number of cells per embryo (1.5×10^3) made things more difficult because a high number of embryos (up to 600) was required for setting an experiment.

Although the culture of carrot embryos should proceed in this work in order to have an assessment of the nutritional and other requirements of the embryonic cells, their culture was attempted directly because as already mentioned the factors controlling growth and morphogenesis in tissue and cell cultures of carrot have been investigated in the past in full detail.

B. MATERIAL AND METHODS

a. Culture of carrot embryo cells

a-1. Sterilization of carrot seed

Carrot seed was rinsed by magnetically stirring it several times in distilled water until the water remained clear. After rinsing, the seed was infiltrated under vacuum for 3 min. and sterilized with NaOCl, 4% available chlorine plus 1% Tween 20, for 20 min. During sterilization the seed was magnetically stirred at the lowest possible speed. Sterilized seeds were rinsed with sterile water and kept soaked overnight in a sterile jar. To find out if sterilization affected embryo viability the TTC test was carried out in a sample of at least one hundred seeds taken after sterilizing and rinsing them.

a-2. Sterilization and aseptic manipulations

Glassware, like small beakers, 10ml glass vials, graduated glass tubes plugged with non-absorbent cotton wool, stainless steel wire meshes, plugged Pasteur pipettes, 5ml glass hypodermic syringes and their needles, Swinnex holders and 0.22 μ m pore size filters for sterilizing liquid media and other solutions, forceps, spatulas and scalpels were wrapped in aluminium foil or put in paper bags and autoclaved for 15 min. at 15lbs/sq. in. and at 121°C.

Agar media were sterilized in glass bottles and adjusted to pH 5.4-5.5 before the agar was added and before autoclaving. Liquid media, enzymic solutions for maceration and 0.6M mannitol for the postdigestion of the macerated embryos were filter sterilized.

Aseptic manipulations were carried out in a laminar flow cabinet which was surface sterilized internally with 70% ethanol and allowed to run for at least 15 min. before manipulations started. Instruments were periodically sterilized with 70% ethanol and flamed. Others like a stereoscope and some other equipment like racks were also surface sterilized with 70% ethanol before they were inserted in the laminar flow cabinet.

a-3. Isolation of carrot embryo cells

After the embryo viability of the sterilized seed was checked, seeds were transferred to a glass Petri dish containing water and with the help of a dissecting stereoscope, forceps and of a scalpel, carrot embryos were squeezed out aseptically at a rate of about one hundred per hour. Every twenty isolated embryos were transferred to the same 10ml glass screw-top vial. When the required number of embryos was obtained the water was removed by carefully using a Pasteur pipette and replaced with 1% filter sterilized Macerozyme solution (pH 5.7) containing 0.6M mannitol. Embryos were then treated according to the procedure described in 2B, a-1. under aseptic conditions. After postdigestion they were transferred in 5ml of nutrient medium containing 0.6M mannitol and sheared immediately. The resulting cell suspension was poured through a double 45 μ m steel mesh to remove any debris, the cell viability was assessed with 0.25% Evan's blue and the cell number was calculated.

a-4. Culture media

The Lin and Staba (1961) medium as modified by Fujimura and Komamine (1975)(Appendix 2) was used for the culture of carrot embryo cells. Stock solutions concentrated 100-1000 times were prepared separately for each substance and stored in glass bottles at 4°C in the dark. Fresh stock solutions were prepared every two months for the inorganic components and every one month for the organic. Aliquots were used to prepare the media which were made up to the final volume with single distilled water. Sucrose was added at 2% unless it is otherwise stated. The agar used to solidify culture media was Oxoid Agar No. 3.

Thermolabile growth regulators were not added to the medium after autoclaving but the inorganic components prepared at half the final volume were sterilized with agar and mixed with the filter sterilized organic components also prepared at half of the final volume. The pH of agar and liquid preparations was adjusted before sterilization to 5.4-5.5.

For experimental uniformity reasons the same procedure was followed for the preparation of agar media every time whether or not they contained any thermolabile constituent.

a-5. Agar cultures

An aliquot (0.2ml) of cell suspension diluted with medium containing 0.6M mannitol was either mixed with agar medium at 30-35°C or plated on the top of an agar medium layer before it was solidified (Bergmann, 1960). 1ml of diluted cell suspension was mixed with 1ml agar medium of the same composition containing no mannitol in a 50mm plastic Petri dish. The Petri dish was shaken for a more uniform cell dispersion within the 1mm thick layer formed.

For plating the cells on the top of a 1mm thick agar layer 2ml of agar medium containing 0.15M mannitol were poured in a 50mm Petri dish and while the medium was solidifying 1ml of the cell suspension was dispersed drop by drop to cover as evenly as possible the top surface.

The final agar concentration in the culture media was 0.6% and the final mannitol molarity was 0.3M. Petri dishes when cool, were sealed with parafilm to avoid desiccation. They were marked and put in plastic boxes with a wet piece of paper on their bottom for better control of humidity.

During plating much care was taken so as never to expose the cells to temperatures in excess of 35°C during their dispersion in the agar medium and to plate with minimum delay.

a-6. Stationary liquid cultures

Plastic bottles 6.5 x 3.7 x 2.0cm (Sterilin products, 43-45 Broad Street, Teddington, TW11 8Q2, U.K.) were used for stationary liquid cultures. An aliquot of cell suspension was diluted with medium containing 0.6M mannitol and was mixed with equal volume of the same medium without or with growth regulators double concentrated. The total volume of the medium was calculated to 2.5ml so that 1mm deep layer was formed. Plastic bottles were marked and put in plastic boxes with a wet piece of paper on their bottom. Nutrient media were filter sterilized.

a-7. Culture conditions

Petri dishes and bottles were incubated at $24 \pm 2^{\circ}\text{C}$ under light (approximately 1,000) lux provided by white cool fluorescent lamps or dark and observed through their base with an inverted microscope. Observations were made every two days for the first ten days and every five days later. With agar cultures positions of interest were marked so that the same area could be observed repeatedly. There were three Petri dishes or bottles per treatment and the results were recorded from six fields of each Petri dish or bottle. The cell density was 4×10^4 cells/ml unless otherwise stated.

b. Carrot embryo culture

For the culture of carrot embryos the Fujimura-Komamine (1975) medium was used. Embryos were originally cultured in 2ml of liquid medium in 50mm plastic Petri dishes. Because of the long-term culture the 2ml of nutrient medium were not enough while the culture in 4ml nutrient medium was inconvenient and contamination occurred very often. To possibly overcome these problems embryos were cultured in 2ml of liquid medium poured on the top of a 1mm thick agar medium layer. The agar medium was prepared as described in a-5. and 2ml of it were poured in a 50mm Petri dish and left to solidify forming a 1mm thick layer. To 1ml of liquid medium containing the isolated embryos, 1ml of medium of the same composition without or with the growth regulators at double concentration was added and the whole was poured on the top of the agar medium. Agar and liquid media in the Petri dish were of exactly the same composition. Agar was incorporated at 0.6% and sucrose at 2%. The pH of the media was adjusted to 5.4-5.5.

Petri dishes were sealed with Parafilm and incubated under approximately 1,000 lux light or dark at $24 \pm 2^{\circ}\text{C}$ temperature. There were three Petri dishes per treatment each one containing 15 embryos. Observations were made weekly with an inverted microscope. During observation Petri dishes had to be handled very carefully because if the liquid medium was coming in touch with the parafilm through the gap between the lateral sides of the base and the lid of the dish, contamination followed. For this reason culture in 4ml of liquid medium was inconvenient and contamination occurred very often.

C. RESULTS

a. Carrot embryo cell culture

a-1. Preliminary experiments

Preliminary experiments were established in order to compare the growth responses of single carrot embryo cells when they were plated on top of an agar and when they were dispersed in the agar. Plating cells on top of the agar layer would be easier than dispersing them. These preliminary experiments involved the culture of zygotic embryo cells in the Fujimura and Komamine (1975) medium supplemented with IAA, 2,4-D and combinations of them with kinetin.

Cells plated on top of the agar layer remained undivided. Only very few cells which were steeped into the agar divided and formed very few colonies. Cells dispersed in the agar layer responded mainly in two ways. About 25% of them gave rise to embryoids, i.e. globular structures some of which continued their development towards embryoids and about 45% formed colonies i.e. a callus mass (Fig. 1). 30% of the cells remained undivided. From the undivided cells however approximately 20% became larger. This increase in size was accompanied by a change in the morphological appearance of the cells, which from spherical became elongated and lost their granular appearance due to their big internal organelles.

a-2. Effect of oxygen

This experiment was based on the observation that single carrot embryo cells did not give any growth response when plated on top of an agar layer while they responded when they were dispersed in the agar. There were three treatments: 1) Cells plated on top

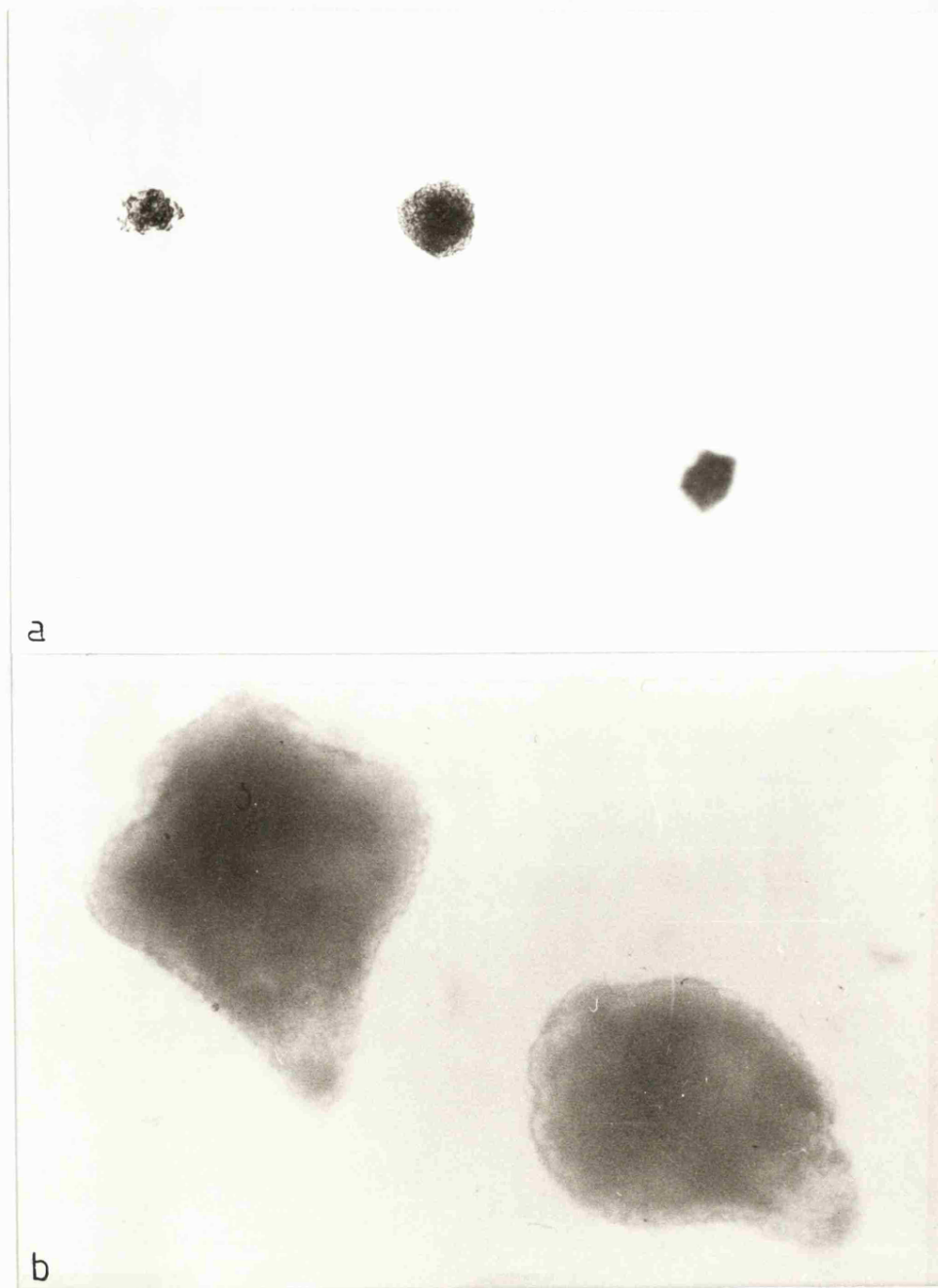


Fig. 1: a. Callus on the left and a globular embryoid in the middle
b. Late globular embryoids. All of them derived directly from
single carrot cells isolated straight from zygotic carrot embryos
var. Chanteney Red Core and cultured in the agar medium of
Fujimura and Komamine (1975) under low light intensity at
 $24 \pm 2^{\circ}\text{C}$ (a x 1.4, b x 5).

Table 1

Percentage of single carrot embryo cells var. Chanteney Red Core which gave rise to embryoids or callus or remained undivided when plated on top or dispersed in different depth layers of agar medium of Fujimura and Komamine (1975) lacking any growth regulators.

Treatment	Embryoids	Callus	Undivided cells
Cells plated on top of agar	0.0	0.0	100.0
Cells dispersed in 0.5mm agar layer	31.3	39.9	28.7
Cells dispersed in 1mm agar layer	16.4	52.0	31.4

of agar. 2) Cells dispersed in a 0.5mm agar layer. 3) Cells dispersed in a 1mm agar layer. For the formation of the 0.5mm agar layer 0.6ml of agar medium was mixed with 0.6ml of liquid medium containing the cells. 1.2ml was the least quantity of medium which could cover the whole surface of a 5cm Petri dish forming a layer of 0.5mm depth.

The results obtained are shown in Table 1. There were significant differences among the treatments showing that the depth of plating in agar has an effect on the type of growth response of the single carrot embryo cells. Organized growth i.e. embryoid formation was optimum when cells were dispersed in 0.5mm agar layer. Again cells which were plated on top of agar remained undivided and only very few cells which were steeped into the agar grew towards callus.

The effect of the depth of cell plating on the growth of single carrot embryo cells was attributed to the different aeration conditions prevailing at different depths of the agar layer and particularly it was attributed to the level of oxygen which obviously was decreasing with the depth of the agar layer.

a-3. Effect of growth regulators

The growth regulators, auxins and cytokinins selected for this study were the IAA, 2,4-D, kinetin and zeatin. IAA and kinetin were used at 1.5mg/l each, 2,4-D at 0.1mg/l and zeatin at 0.02mg/l. These concentrations were chosen arbitrarily since isolation of carrot embryos at a large scale was a limiting factor not allowing

experiments with many replicates in which a factorial interaction of the growth regulators at different levels could be studied. Furthermore concentrations of IAA and kinetin at the same concentrations have been used successfully by Konar and Nataraja (1965) for inducing embryo formation in agar cultures of Ranunculus sceleratus cells.

Table 2 shows the percentages of single carrot embryo cells which responded to embryoid and callus formation or remained undivided. Among the different treatments there were no significant differences. Cells were cultured in a 0.5mm agar layer.

a-4. Cell density

This experiment was planned in order to find out what was the minimum effective cell density in agar cultures of single carrot embryo cells. If the minimum effective cell density was to be proved properly low then from cells isolated from a single zygotic embryo, adventive embryos and colonies or clones could possibly be obtained. It would therefore be interesting to compare such clones and the plants to which adventive embryos could probably develop.

Table 3 shows that cells could not divide and grow at densities lower than 10^4 cells/ml. For cell densities higher than 10^4 cells/ml inclusive there were no significant differences in respect to the percentage of cells which gave rise to embryoids or callus. The cell density of 8×10^4 cells/ml proved to be rather high. Observation of individual cells and counting of embryoids, colonies and cells was occasionally difficult at this density.

Table 2

Percentage of single carrot embryo cells var. Chanteney Red Core which gave rise to embryoids or callus or remained undivided when cultured in the Fujimura and Komamine (1975) agar medium supplemented with growth regulators^{*} and combinations of them.

Treatment	Embryoids	Callus	Undivided cells
Control	30.1	38.7	31.0
IAA	28.7	40.0	31.1
IAA + kin	28.2	42.4	29.2
2,4-D	31.6	38.8	29.4
2,4-D + kin	28.7	40.2	30.9
Zeatin	32.1	30.0	29.7

* For their concentrations see text.

Table 3

Percentage of single carrot embryo cells var. Chanteney Red Core which gave rise to embryoids or callus or remained undivided when plated at different cell densities in Fujimura and Komamine (1975) agar medium lacking any growth regulators.

Cell density	Embryoids	Callus	Undivided cells
0.5×10^4	0.0	0.0	100.0 *
1×10^4	21.0	47.17	31.8
2×10^4	20.3	47.87	31.8
4×10^4	18.9	48.60	32.4
8×10^4	22.6	44.71	32.6

* A small percentage of cells divided but not any endryoid or callus was raised from them

Cells in this experiment were plated in a 1mm agar layer.

a-5. Embryo development

The purpose of this experiment was to assess the percentage of embryoids which reached different stages of development. This assessment was carried out with carrot embryo cells cultured under different conditions of light i.e. low light intensity as described in Material and Methods and with different plating depths as described in a-2.

Table 4 shows the percentages of single carrot embryo cells which gave rise to embryoids and calluses or remained undivided. In respect to embryoid formation the differences between the different plating depths and between the different light conditions were significant. The highest percentage was obtained under low light intensity and with cells dispersed in 0.5mm deep agar layer.

Although the percentage of single cells which gave rise to embryoids was affected by the different treatments involved in this study the percentage of embryoids which reached different stages of development did not vary significantly from one treatment to another (Table 4a).

The growth of embryos was very fast during the first four days and slow later. Single cells divided rapidly and gave rise to globular units. While the major percentage of these units remained as globular (Table 4a) some others continued their development and obtained the heart shape and some of them differentiated cotyledons (torpedo stage). In practice very few reached the cotyledonary stage and, in the presence of growth regulators, some torpedo

Table 4

Percentage of single carrot embryo cells var. Chanteney Red Core which gave rise to embryoids or callus or remained undivided when plated on top or dispersed in different depth layers of agar medium of Fujimura and Komamine (1975) lacking any growth regulators under different light conditions.

Type of Growth	Embryoids		Calluses		Undivided cells	
Treatment	Light	Dark	Light	Dark	Light	Dark
Cells plated on top of agar	0.0	0.0	0.0	0.0	100.0	100.0
Cells dispersed in 0.5mm agar layer	27.9	12.3	43.7	54.7	28.2	32.8
Cells dispersed in 1mm agar layer	18.6	10.4	50.2	56.6	31.0	32.9

Table 4a

Stage of development of embryoids, raised from single cells isolated from carrot embryos var. Chanteney Red Core and cultured in the Fujimura and Komamine (1975) medium lacking growth regulators.

Treatments	Stage of embryo development															
	Globular				Heart		Torpedo				Cotyledonary					
	Light		Dark		Light	Dark	Light	Dark	Light	Dark	Light	Dark				
	1*	2*	1	2	1	2	1	2	1	2	1	2				
Cells plated on top of agar	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
Cells dispersed in 0.5mm agar layer	79.2	22.1	78.5	9.9	14.6	4.0	15.7	1.9	5.0	1.4	4.9	0.6	1.0	0.2	0.8	0.1
Cells dispersed in 1mm agar layer	81.1	15.1	81.8	8.5	14.4	2.7	15.9	1.6	3.1	0.5	2.2	0.2	1.2	0.2	1.1	0.1

* 1: Percentage of embryoids which reached different stages of development in respect to the total number of embryoids.

2: Percentage of embryoids which reached different stages of development in respect to the initial number of single cells.

embryoids proliferated into callus.

Cells which gave rise to embryoids were the smaller in comparison to those which gave rise to callus or remained undivided. With embryoids after repeated cell divisions, cells continued to be small in size and similar in appearance with those from which they originated i.e. similar to the smaller cells of the zygotic embryos. Calluses initiated from single carrot embryo cells consisted of larger cells in comparison to those from which they were raised off. It was out of the purpose of this work to study the cytological changes which were occurring during embryogenesis and callogenesis.

Somatic embryos raised from single cells were small in size and probably this was because of the small size of the cells from which they were consisted of and/or because of their origin straight from zygotic embryo cells. Throughout the initiation and development of embryoids no intervening callus phase was observed and no suspensor-like structure was detected in embryoids of any stage of embryonal development.

Observations in this experiment were recorded after 30 days culture.

a-6. Effect of osmotic concentration

Certain environmental factors like light and total osmotic concentration of the medium seem to be related to induced morphogenetic responses. In a-5 it was shown that light had an effect on the percentage of single cells giving rise to embryoids but it had no effect on the development of the produced embryoids. Measurements

of the osmotic pressure of the ovular fluids indicated that embryos are bathed by relatively strong solutions (Ryczkowski, 1962). From the works of Ziebur and Brink (1951), Rijven (1952), Rietsema, Satina and Blakeslee (1953) and Norstog (1961) it has been observed that isolated immature zygotic embryos profited from culture in more concentrated media with increased concentrations of sucrose. The greater concentration fostered the completion of embryonic development, whereas plantlets emerged when the sucrose level was lowered.

This experiment was planned on the idea that culturing embryogenic cells under conditions resembling closer the conditions under which embryos are formed in the ovule, would result in the increase of the percentage of embryoids reaching complete development. Sucrose was included in the medium at 2, 4, 8 and 12%. The increase of the sucrose concentration in the medium up to 12% approximates the known osmotic value of certain ovular fluids and also that of certain other media which have been used to cultivate excised zygotic embryos (Ammirato and Steward 1971). The experiment was repeated twice. The first time 0.3M mannitol was incorporated into the medium while in the second experiment it was omitted. From the two experiments similar results were obtained and this indicated that stabilization of cells with 0.3M mannitol was not an absolute requirement. Table 5 shows the results obtained with the first experiment. Higher sucrose concentrations did not have any effect on the completion of embryoid development.

Table 5

Stage of development of embryoids, raised from single cells isolated from carrot embryos var. Chanteney Red Core and cultured in the Fujimura and Komamine (1975) medium lacking growth regulators and supplemented with different concentrations of sucrose.

Sucrose concentration %	Stage of embryo development							
	Globular		Heart		Torpedo		Cotyledonary	
	1*	2*	1	2	1	2	1	2
2	82.0	26.4	12.8	4.1	3.8	1.2	1.3	0.4
4	76.9	23.2	16.3	4.9	5.7	1.7	0.9	0.2
8	81.4	22.9	14.4	4.0	3.3	0.9	0.8	0.2
12	80.9	23.4	15.4	4.4	3.0	0.8	0.5	0.1

* 1: Percentage in respect to the total number of embryoids.

2: Percentage in respect to the initial number of single cells.

a-7. General observations

In all the previous experiments apart from embryoid and callus formation there were always approx. 20% of cells which did not divide but showed an increase in their size followed by cytological changes. Similar cell size increase has been reported by Blakely (1964) with secondary cultures. In every experiment if the percentage of cells which were expanded was added to the percentage of cells which gave rise to embryos and callus then the total percentage was approx. 90%. This is the same with the percentage of viable embryonic cells after isolation and before culture and indicates 1) that the viability test (Evan's blue, Gaff and O'Okong Ogloa, 1971) used in this work was giving real figures; 2) that almost every cell was capable of growth and division since only 10% of the cells which were assumed dead did not give any growth response.

b. Stationary liquid cultures

Stationary liquid cultures were employed in this study for the simple reason that they were easier to be established than the agar cultures in spite of their disadvantage that the growth of individual cells could not be monitored. Cells were cultured in Fujimura and Komamine (1975) medium supplemented with growth regulators as described in a-3. They were also cultured under light and dark. The cell density in all these cultures was 10^4 cells/ml.

The results obtained with stationary liquid cultures were negative. Embryonic cells could not remain uniformly dispersed,

they came together forming large loosely connected colonies and they were floating. Some cells divided but continuous growth could not be supported. Embryoid formation in stationary liquid cultures was very rare and happened after approximately three month's culture. Embryoids remained globular and did not develop any further.

c. Carrot embryo culture

As already mentioned carrot embryo culture should precede the carrot embryo cell culture because an assessment of the nutritional and environmental requirements of the embryonic tissues for growth and morphogenesis might give useful information for the cell culture in respect to the same objective. The work with carrot embryo cells however was based on the voluminous research to detail on carrot embryogenesis. So this work followed, in order to compare the growth of zygotic carrot embryos, with the responses of carrot embryo cells isolated straight from zygotic carrot embryos.

In the first experiment zygotic carrot embryos were cultured on the Fujimura and Komamine (1975) medium supplemented with IAA 1.5mg/l, 2,4-D 0.1mg/l, kinetin 1.5mg/l and combinations of them. The agar used was the Oxoid Agar No. 3 and the light (approx. 10^3 lux) was provided by white cool fluorescent lamps. In the second experiment zygotic carrot embryos were cultured under exactly the same conditions except that the agar used was Difco Bacto agar and that the light was 200-1000 lux, as already described in Chapter 4B, a-3. Another difference was that the two experiments were set by using seed from different batches. There were three replicates per treatment each one containing 10-15

embryos.

From the two experiments similar and different results were obtained. In the first experiment and in the control (i.e. without any growth regulator) treatment under light embryos developed to plantlets (Fig. 2a). Under dark embryos developed towards etiolated plants while from sloughed off cells many embryos were formed (Fig. 2b). Cells were sloughed off mainly from the area around the root tip. Similar were the responses of embryos under light and dark when in the medium 1.5mg/l IAA was incorporated. The only difference was that embryoids formed from sloughed off cells were visible macroscopically earlier i.e. three months after the culture was set whereas in the control treatment embryoids appeared after five months. In both treatments single cells, cells undergoing division, a few celled aggregates and embryoids could be observed. When the medium was supplemented with 0.1mg/l 2,4-D under light, embryos formed very thick cotyledons and a very thick hypocotyl in the first two weeks and obtained green colour without developing further. Under dark embryos proliferated towards callus. The response of the embryos was very characteristic when the medium was supplemented with 1.5mg/l kinetin. Under light they developed to plants similar to those illustrated in Fig. 2 with the difference that root growth was inhibited, roots remained very short (5-10mm) and obtained a brown colour. Under dark the majority of the embryos formed a barrel-shaped hypocotyl, the root remained undeveloped and the thickened cotyledons seemed to be sitting on the hypocotyl (Fig. 2c). Other embryos had a thickened hypocotyl and cotyledon, while the shoot meristem had the form of a globular outgrowth

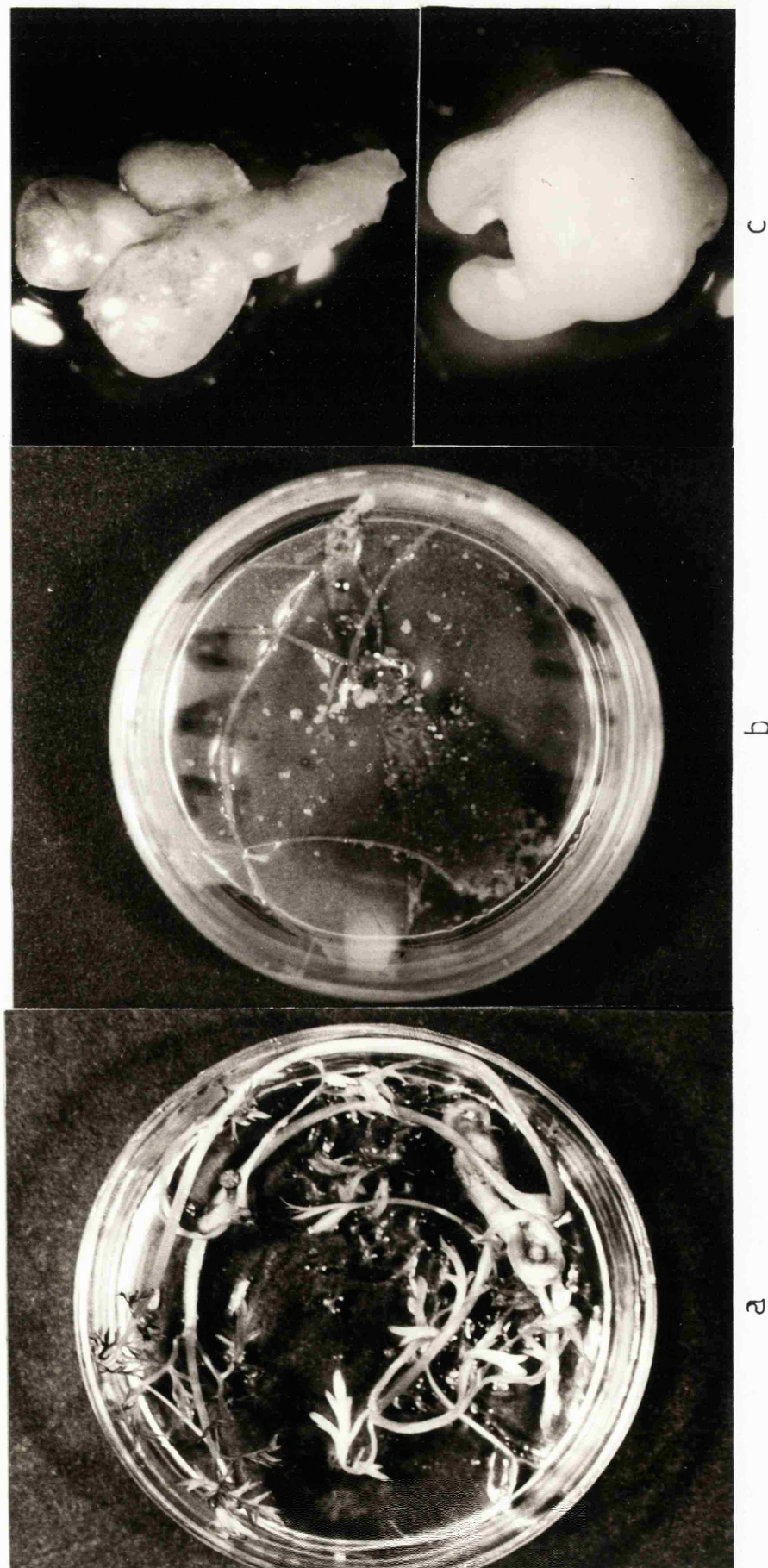


Fig. 2: Zygotic carrot embryos, var. Chanteney Red Core, cultured in the Fujimura and Komamine (1975) medium with or without growth regulators under light or dark at $24 \pm 2^{\circ}\text{C}$. a. Plantlets to which embryos developed under light and in the absence of growth regulators. b. Etiolated plantlets to which embryos developed under dark in the absence of growth regulators and adventive embryo formation from sloughed off cells. c. The barrel-shaped hypocotyl was the main characteristic response of the majority of embryos cultured in the presence of kinetin (lower embryo). A few embryos had thickened hypocotyl and cotyledons and a shoot meristem like a globular outgrowth (upper embryo). a, b x 1.8, c x 2.8.

(Fig. 2c). When the medium was supplemented with 2,4-D(0.1mg/l) and kinetin (1.5mg/l) under light and dark embryos proliferated towards callus. The treatment 1.5mg/l IAA plus 1.5mg/l kinetin was contaminated.

In the treatments where proliferation of the embryos occurred many cells were released and floating free in the medium. These cells which were larger and elongated in comparison to the cells from which originated, showed division, but no organized growth i.e. embryo formation, was observed from them.

In summary there were differences between light and dark treatments and embryogenesis was observed under dark. This was somewhat in opposition with the fact that the same light conditions resulted in higher percentages of single embryo cells giving rise to embryoids in comparison to those obtained under dark.

In the second experiment zygotic embryos responded similarly under light and dark. In control treatments cotyledons grew thick and the hypocotyl as well while the root remained undeveloped. The growth of the cotyledons was bigger in comparison to growth of the hypocotyl (Fig. 3a). From the root and the hypocotyl area of some embryos somatic embryos emerged after approximately five month's culture (Fig. 3b). Many adventive embryos were also formed from freely suspended cells which were sloughed off from growing embryos and from the root and lower hypocotyl area of growing zygotic embryos (Fig. 3c). These embryos (Fig. 3d) covered the major area of the Petri dish. All the somatic embryos formed in these treatments either along the axis of zygotic embryos or from freely suspended



For explanation see over.

Fig. 3: Zygotic carrot embryos, var. Chanteney Red Core, cultured in the Fujimura and Komamine (1975) medium lacking growth regulators at $24 \pm 2^{\circ}\text{C}$. a: Embryos after five months culture. b. A detail of a. Adventive embryos outgrowing along the axis of the zygotic embryo. c. Cells sloughing off from the root and the hypocotyl area of growing zygotic embryos. d. Adventive embryos originating from sloughed off cells from zygotic embryos in culture. a, b, c, d x 3.5.

cells did not pass the torpedo stage in their development.

When 1.5mg/l IAA was incorporated in the medium, embryos responded in similar ways. The cotyledons grew very thick in relation to other parts of the embryo (Fig. 4a). Secondary embryo formation was observed along the hypocotyl and around the root area (Fig. 4a-b) and many embryos were formed from sloughed-off cells (Fig. 4c). The impression, albeit not quantitatively documented, was that this treatment in comparison to control yielded a higher number of embryos which were more advanced in development. Another difference between this treatment and the control was that secondary embryos (embryos formed along the axis of zygotic embryos) and embryos formed from freely suspended cells in this treatment could visually be observed after three months culture in comparison to five for the control.

0.1mg/l 2,4-D resulted in the growth of the cotyledons and of the area of the hypocotyl just below the shoot meristem. Fig. 5a-b shows how zygotic embryos grew in the presence of 0.1mg/l 2,4-D. Fig. 5c which is a detail of the Fig. 5b shows the typical growth of a zygotic embryo in this treatment. Although many cells were released into the medium from the root area of several embryos and these cells showed divisions no embryo formation was observed from them. No secondary embryos were formed along the axis of zygotic embryos either.

In the presence of 1.5mg/l kinetin, embryos responded with a characteristic growth of the hypocotyl like that illustrated in Fig. 6a. Cotyledons grew very little. When IAA and kinetin were

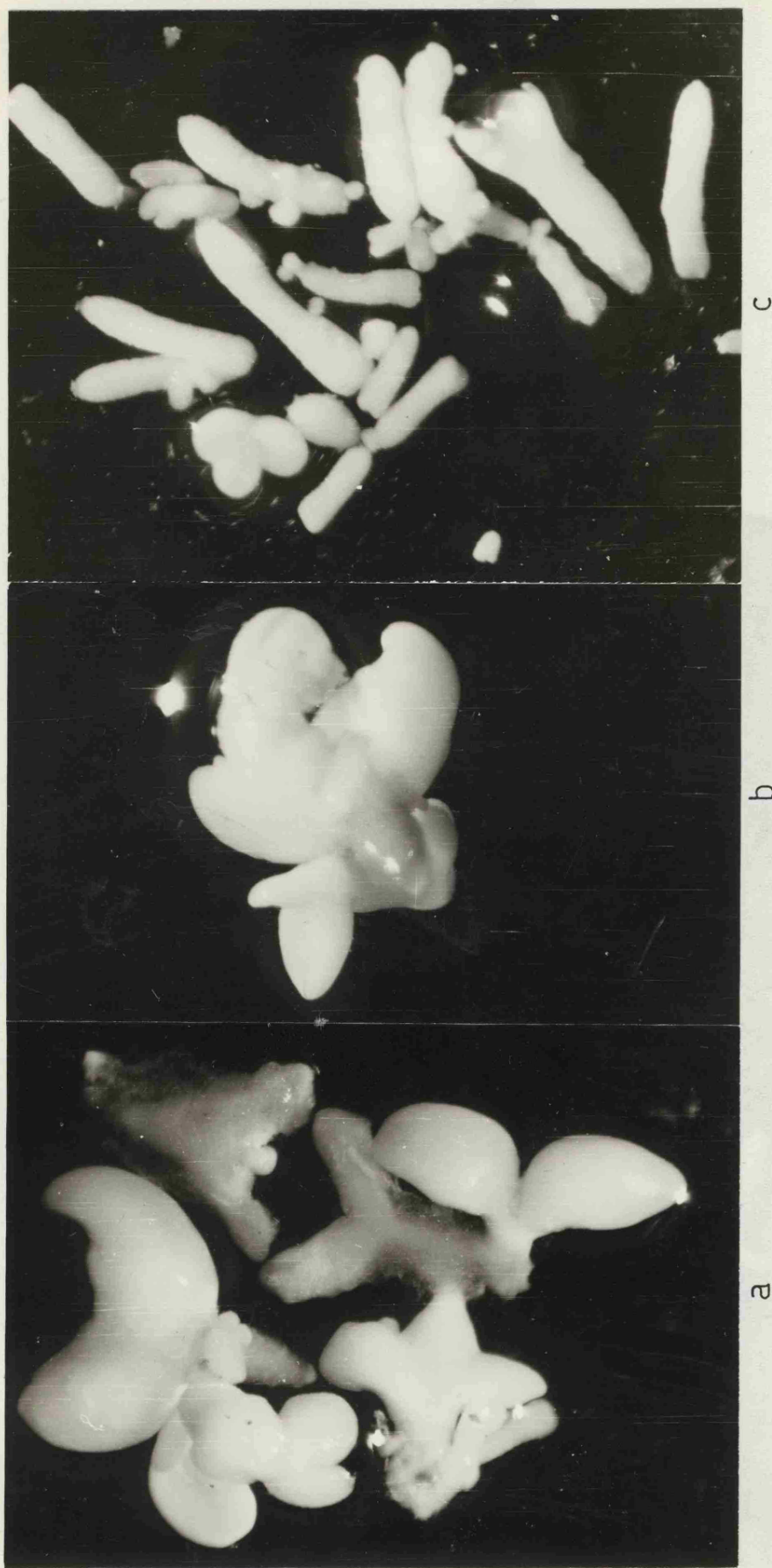


Fig. 4: Zygotic carrot embryos var. Chanteney Red Core, cultured in the Fujimura and Komamine (1975) medium supplemented with 1.5mg/l IAA at $24 \pm 2^{\circ}\text{C}$. a. Embryos after three months culture, showing secondary embryo formation. b. An adventive embryo cotyledonary stage arisen at the base of the cotyledon of cultured zygotic embryo. d. Adventive embryos originated from sloughed-off cells from zygotic embryos. a, b, c x 3.5.

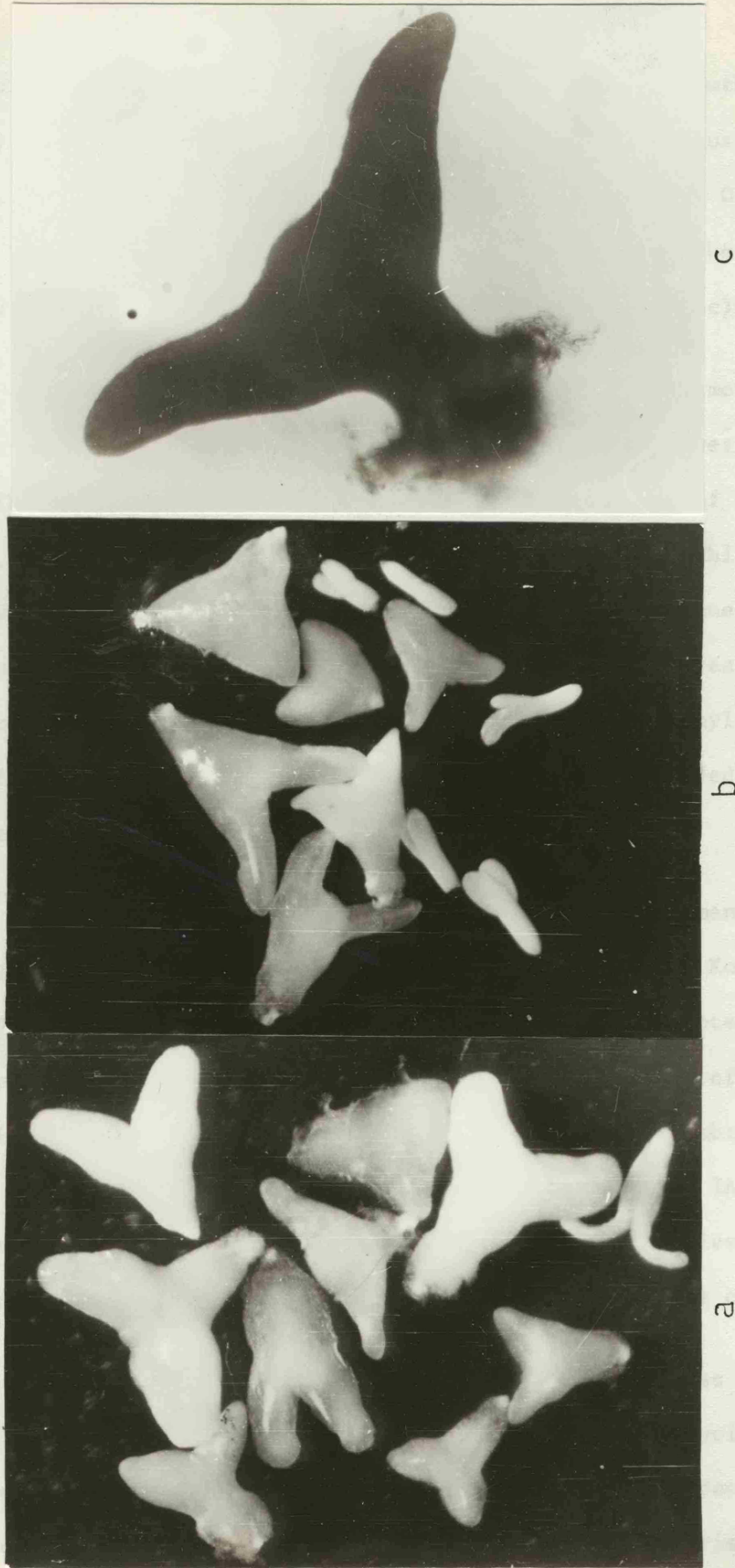


Fig. 5: Zygotic embryos cultured in the Fujimura and Komamine (1975) medium supplemented with 0.1mg/1 2,4-D at $24 \pm 2^{\circ}\text{C}$. a-b Zygotic embryos after three months culture. c. A detail of (a) showing what could be described as representative response of zygotic carrot embryos cultured in the presence of 0.1mg/1 2,4-D a, b x 3.5, c x 7.

combined at concentration 1.5mg/l each simultaneous growth of the cotyledons and the hypocotyl was observed like that illustrated in Fig. 6b. Under the combination of 2,4-D and kinetin at 0.1mg/l and 1.5mg/l respectively cotyledons and hypocotyls of the embryos grew retaining the original shape of the embryo (Fig. 6c).

The general observations of the second experiment except that embryos responded similarly under light and dark were:

- 1) That under every treatment and whatever the growth of the embryo was, it was completed within three weeks of culture. While the growth of embryos was ceased after three weeks embryogenesis was observed after three to five months.
- 2) Under light treatments no embryo became green i.e. no embryo synthesized chlorophyll.
- 3) Among the somatic embryos raised from freely suspended cells several had more than two cotyledons.

The general observations from the two experiments were as follows. Embryogenesis was observed in the Fujimura and Komamine (1975) medium lacking any growth regulator or supplemented with 1.5mg/l IAA. In the presence of 2,4-D in the presence of kinetin either alone or in combination with IAA and 2,4-D no embryogenesis was observed. There should be a reservation about the IAA plus kinetin treatment of the first experiment but nevertheless it was unlikely that embryogenesis would occur in the presence of kinetin.

It is important to mention that in the process of embryo isolation from seed under aseptic conditions some embryoids which were kept in sterile water grew 5mm long with fully expanded cotyledons but there was no obvious growth of the shoot meristem. This

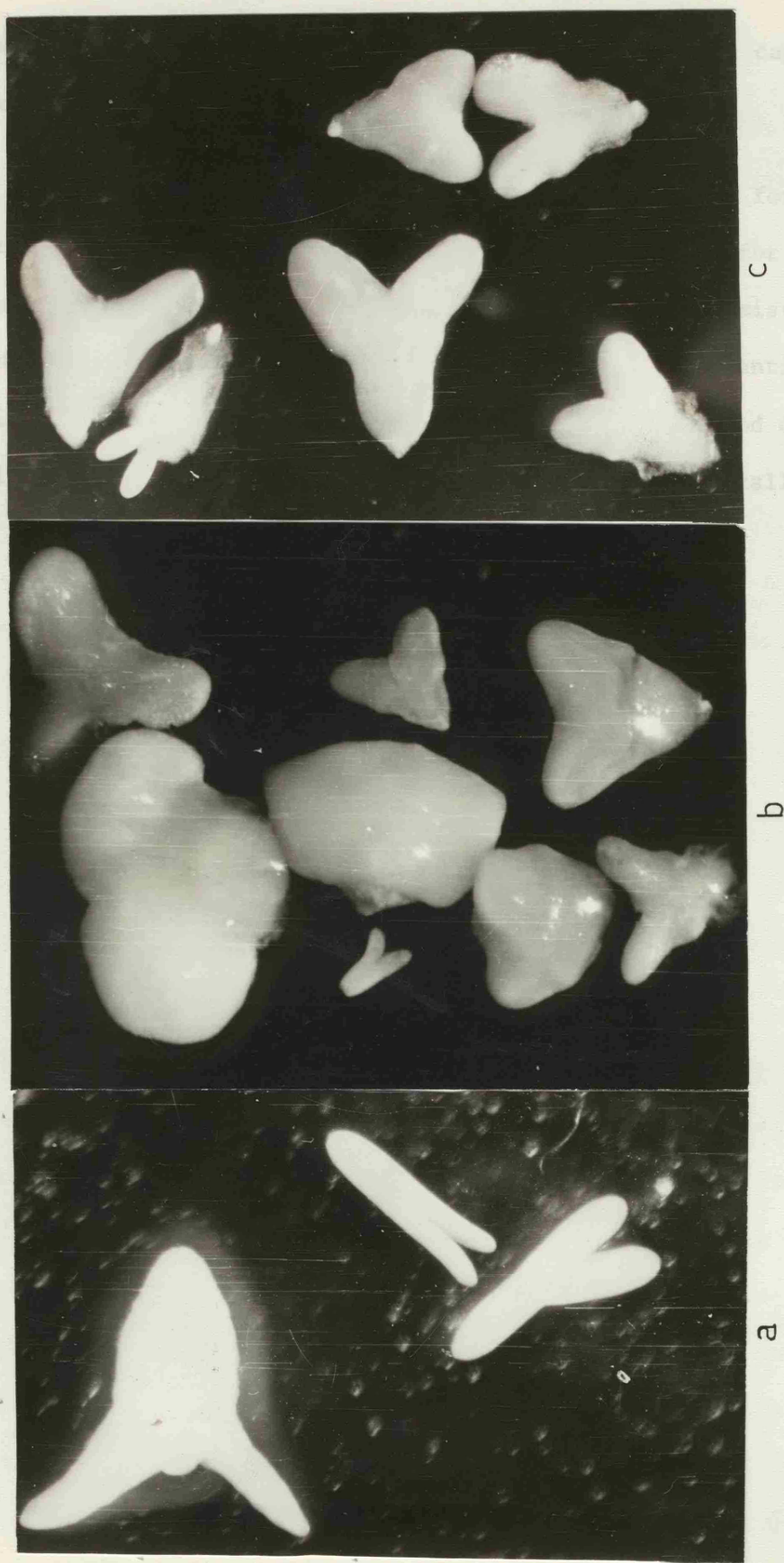


Fig. 6: Zygotic embryos cultured in Fujimura and Komamine (1975) medium supplemented with 1.5mg/l kinetin (a), 1,5mg/l kinetin + 1, 5mg/l IAA (b) and 1.5mg/l kinetin + 0.1mg/l 2,4-D(c). On the left an embryo after three months culture in comparison to freshly isolated embryos b-c. Embryos after three months culture. a,b, c x 3.5.

was an indication that carrot embryos have an innate capacity of certain growth.

Digestion of adventive embryos, which were formed in the two experiments described above, with 1% Macerozyme for 2h at 32°C and 24h postdigestion in 0.6M mannitol, showed the existence of a cuticle surrounding the embryo. Maceration of adventive embryos into single cells was possible by following the method described in Chapter 2B, a-1, but it was not studied systematically.

D. DISCUSSION

Carrot embryogenesis is a very well-known process with secondary liquid cell cultures. Cell populations originated from an explant and grown in media containing auxin, give rise to embryoids when transferred to the same media lacking the auxin (Halperin 1966). In this work carrot cells isolated straight from zygotic embryos developed directly to embryoids when cultured in a medium lacking the auxin. The origin of the cells, meristematic and differentiated cells isolated directly from zygotic embryos, and the use of Fujimura and Komamine (1975) medium, a well-established medium for carrot embryogenesis are possibly the two main factors for the development of the cells to embryos. The shearing of the embryos in the culture medium could be considered as another factor. Maceration of tissues into single cells is a process during which the resulting leaky cells lose metabolites. In these experiments carrot embryos were sheared in the culture medium and this minimised loss of metabolites.

Carrot embryo cells did not give any growth response when plated on top of an agar layer, while they responded when they were dispersed in the agar. Street (1977,b) obtained less satisfactory and more variable results from distributing the cells in a liquid film over the agar surface than incorporating the cells into the body of the thin film of agar. In this work the different responses of cells when they were plated at different depths in respect to the agar layer were attributed to an oxygen gradient from the surface to the bottom of the agar layer. In 0.5mm deep agar layer the highest percentage of cells giving rise to embryoids was observed. Probably in such a layer most of the plated cells were under oxygen conditions

favouring embryogenesis. Further supply of oxygen (cells plated on top) did not favour any growth of cells while with deprivation of oxygen supply (cells dispersed in 1mm deep agar layer) the percentage of cells giving rise to embryoids was decreased.

The view that the different growth responses could be attributed to the level of oxygen is supported by the work of Kessell and Carr (1972) and Kessell et al. (1977) who found that the oxygen concentration has an effect on the growth and differentiation of carrot cell cultures. According to Kessell and Carr (1972) for suspension cultures of carrot there is a critical level of dissolved oxygen concentration. Below this level, dry weight increased linearly, cell number increased exponentially and "meristem-like" conditions were created in the culture which favoured the formation of plantlets by embryogenesis. Conditions above the critical level resulted in exponential increase of the cell dry weight and cell number and favoured plantlet differentiation by rhizogenesis.

The data of this work and those presented by Kessel and Carr support White's early suggestion that oxygen gradients may be significant in regulating organogenesis.

By plating cells at different cell densities it was found that 10^4 cells/ml was the minimum effective cell density while cell densities higher than 4×10^4 cells/ml in some cases prevented the observation of individual cells and made counting difficult.

Thinking that each zygotic carrot embryo consists of approximately 1.5×10^4 cells, the obtention of colonies and adventive embryos

from single cells isolated from a single zygotic carrot embryo is possible by dispersing the cells in a 0.5mm agar layer. The reduction of the total number of cells per Petri dish to the cell number per embryo was one more reason in this work for using agar layers 0.5mm in depth.

According to Halperin (1970, 1973) embryonic cells are characterized by the ability to grow in the absence of exogenous hormones. In this work cells isolated from zygotic embryos when cultured in the absence of exogenous regulators responded in three different ways. Firstly a percentage of them gave rise to organized growth, i.e. embryo formation, secondly another percentage gave rise to visible colonies and thirdly another percentage was increased in size. The fact that cells of embryo origin responded in three different ways when cultured under **similar** nutritional and environmental conditions should be attributed to the different degrees of differentiation the cells represent.

The growth responses of embryo cells were the same when they were cultured in the same medium supplemented with growth regulators as described in a-3. This indicated that embryogenesis was not inhibited by the growth regulators used in this work. There are conflicting reports on the effect of growth regulators on the embryogenesis. Fujimura and Komamine (1975) reported that embryogenesis in carrot cell suspensions was inhibited by 2,4-D and IAA at relatively low concentrations but zeatin had a promotive effect on embryogenesis only at a narrow range of its concentration. Kameya and Uchimiya (1972) reported the differentiation of embryoids from isolated pro-

toplasts of carrot in the presence of 1% kinetin. Kato and Takeuchi (1963) starting from single cells of carrot root reported that embryos were differentiated in a medium supplemented with 1 or 10 ppm IAA without transfer to auxin-less or to a lowered auxin concentration medium. Differentiation of embryoids in the presence of IAA and kinetin (1.5ppm each) has also been reported by Konar and Nataraja (1965) in agar cultures of Ranunculus sceleratus cells. The expression of totipotency of zygotic embryo cells in the presence of various growth regulators and in their absence should be attributed to the strong competence of these cells isolated at a stage of plant development which is very close to the zygote.

Transfer from an auxin containing medium to another lacking the auxin or containing it at a lower concentration (sequential effect) is the usual practice for induction of embryoid formation in suspension cultures (Halperin, 1966; Steward et al., 1967). In this work on embryo formation from single carrot embryo cells no transfer to media varying in respect to auxin concentration was necessary. In the works reported by Kato and Takeuchi (1963) and Konar and Nataraja (1965), as well, embryos were formed in the same medium without any transfer to another.

Cells isolated from zygotic carrot embryos divided rapidly during the first four days (first phase) giving rise to globular embryoids. Cell division and growth was very slow after the fourth day (second phase). These results generally agree with those obtained by Fujimura and Komamine (1980) who working with secondary

cell suspensions of carrot found that with carrot embryogenesis three phases could be recognised in each of which the rate of cell division was different. In the first phase which lasted 0-3 days cells divided slowly. In the second phase (3 to 4 days) cells divided very rapidly and globular embryos were formed. In the third phase (4 to 6 days) cell division occurred at a slower rate than the second phase. In this work cells were capable of active cell division immediately after their plating in agar. As already mentioned in Chapter 2B, a-4.1, cells, released from embryos sheared and postdigested in 0.6M mannitol, were already undergoing division. Except the embryonic nature of the cells which favours active cell division, the previous starvation of the cells in 0.6M mannitol might be an extra reason for their capability of rapid division immediately after their isolation and culture.

Single carrot embryo cells developed to embryoids without any intervening callus phase. Similar results were obtained by Kato (1968). In his work epidermal cells could immediately join the adventive embryogenesis without passing through the stage of callus in spite of the recognition that in some cases epidermal cells passed through the state of loose callus before they entered into the process of embryogenesis. Another common character of his work in respect to this work is that while some epidermal cells embarked upon embryogenesis others divided and formed callus. One difference was that in this work from a single cell only a single adventive embryo was formed while Kato reported that in some cases from one epidermal cell more than two adventive embryos were derived. Embryo formation from single carrot embryo cells (isolated

from secondary cultures) was also reported by Backs-Hüsemann and Reinert (1970). In their work though the development of embryoids from the single cells was not direct. Between the single cell stage and adventive embryos there was an intervening callus phase.

About 30% of single carrot embryo cells developed to embryoids when cultured in the Fujimura and Komamine (1975) medium. Although this medium allowed the growth of cells to embryoids it could not support their further development up to the cotyledonary stage and/or to plantlets. If this was possible then the culture of cells isolated from a single zygotic embryo would be a powerful tool for plant physiologists because apart from the single cell derived clones, plants of single cell origin could be compared for their genetic uniformity. Derivation of carrot plantlets through adventive embryogenesis was reported by several workers (Kato and Takeuchi, 1963; Halperin and Wetherell, 1965; Sussex and Frei, 1967; Grambow et al., 1972). Plantlets were also derived through adventive embryogenesis in Raunuculus sceleratus cell cultures (Konar and Nataraja, 1965). In the works of Kato and Takeuchi (1963) and Konar and Nataraja (1965) the characteristic was that plantlets were developed from embryos on the same medium in which the cells were originally cultured.

Between embryo initiation and embryo development to plantlets the second would be easier. After the formation of embryos of single cell origin is achieved their further development to plantlets could be promoted either by modifying the medium or by transferring the embryo into another medium.

Increase of the osmotic concentration of the medium did not have any effect on the completion of embryoid development. Ammirato and Steward (1971) reported that in carrot cell cultures the higher concentrations of sucrose, mannitol or sorbitol kept the embryos small and more similar in appearance to zygotic embryos at the cotyledonary stage. They also reported the appearance of numerous secondary adventitious embryoids along the entire axis of the smaller embryos developed at higher osmotic concentrations. In this work the embryoids which were formed were small in size as mentioned in a-5, and higher osmotic concentrations did not result in formation of embryoids of smaller size. No formation of secondary embryoids was also observed along the axis of embryos under any osmotic concentration.

It was observed that cells which gave rise to embryoids were the smaller whereas those which gave rise to callus were larger and probably more differentiated. The conclusion was that between cells giving growth to embryoids or callus there are morphological differences and differences in the degree of differentiation. This is in agreement with Jones (1974) who reported that cells, which are recognised as precursors of embryogenesis and readily give rise to spherical embryos when the auxin content is reduced, are small and in general it is rare for them to become enlarged and vacuolated.

The percentage of cells which gave rise to embryoids was affected by the light treatment. Higher percentages were obtained under low light intensity than dark. Kessell and Carr (1972) reported that morphogenetic responses of cultured cells of carrot

occurred in continuous light, alternating 12h light-dark cycles and in complete darkness, but there were strong indications that embryogenesis occurred more readily under alternating light. In this work it was difficult to explain why the percentage of cells from which embryos were formed was higher under light conditions. Further experimentation involving light intensity, spectral quality and length of the light exposure period should be carried out, before any explanation is given on the morphogenetic effects of light at cellular level with carrot cultures.

From the two types of cell culture and stationary liquid cultures used in this work, agar cultures gave good results, while in stationary liquid cultures cells responded very rarely in respect to growth and morphogenesis. Brown et al., (1976) reported that liquid media support embryogenesis better than agar media but they did not provide any experimental evidence justifying this view. The best justification though for this view is the fact that the majority of the studies concerning carrot embryogenesis have been carried out using liquid media or better by using agitated liquid cultures. Stationary liquid cultures are not known as a means for studying problems on carrot embryogenesis.

Zygotic carrot embryos responded in various ways when cultured on the same medium under different conditions. Some responses however were consistent under the different conditions employed in each of the two experiments. For example, embryogenesis from freely suspended cells was observed under dark in the control treatments and in the presence of 1.5mg/l IAA. Embryogenesis either from freely suspended cells or along the axis of the zygotic embryos

was observed after five months in the control treatments and after three months in treatments containing 1.5mg/l IAA. Kinetin caused an inhibition of the root development and characteristic growth of the hypocotyl.

In this work adventive embryos were formed along the axis of zygotic carrot embryos. Ammirato and Steward (1971) reported formation of adventive embryos along the axis of somatic embryos developed in media with high osmotic concentration. McWilliam et al. (1974) reported the initiation of additional embryoids from the epidermis of some of the embryoids developing in culture.

Formation of adventive embryos from the cotyledons of zygotic embryos of Biota orientalis and of Ilex aquifolium was reported by Konar and Oberoi (1965) and Hu and Sussex (1971) respectively. A common feature of the embryoids developed along the axis of zygotic carrot embryos in this work and of the embryoids developed on cotyledons of Ilex aquifolium zygotic embryos was that they were formed on media lacking growth regulators.

The general conclusion from the culture of cells isolated straight from zygotic carrot embryos and the culture of intact zygotic embryos was that embryonic tissues and cells responded similarly on the same medium and in respect to morphogenesis. There were of course some differences, e.g. isolated single cells gave rise to embryoids in the presence of 2,4-D and kinetin while zygotic carrot embryos and freely suspended cells which were sloughed off from the zygotic embryos did not show any embryogenesis in the presence of the same growth regulators. The different response of zygotic embryos should be attributed to an interaction of the exogenously supplied

regulators with the endogenous regulators of the zygotic embryos which were probably diffused into the culture medium and similarly affected the growth and morphogenetic responses of the freely suspended cells.

CHAPTER 4

MORPHOGENESIS IN APPLE EMBRYONIC TISSUE CULTURES

A. INTRODUCTION

The further purpose of this study is to obtain an approximate estimation of what the nutritional and hormonal requirements of apple embryo cell suspensions are in respect to morphogenesis and in vitro regeneration. An assessment of the same requirements of the embryonic tissues of apple could give this approximate estimation before starting a study on the primary embryo cell cultures. It could also give a new dimension to the mass propagation of apple and provide new knowledge on the morphogenetic processes occurring with apple embryonic tissues.

The new dimension is justified by the fact that embryonic tissues of apple have not so far been used as a source for plant regeneration. Apomixis (Sax, 1959; Campbell and Wilson, 1961; Schmidt, 1970) occurring in several apple varieties, the probability and/or possibility of experimentally inducing apomixis and the fact that viruses are not usually transmitted through the seed are in favour of studying apple regeneration from embryonic tissues. Furthermore production of a certain number of plantlets from a single embryo and their consequent propagation by means of tissue culture could result in the production of clones on which the same variety when budded or grafted would produce uniform trees.

The knowledge obtained by such a study could be important as a source for basic research and as a contribution to ways of mass propagation of apple which are possibly shorter, more advantageous from the point of view of genetic stability and more economical.

According to Skirvin (1980) the induction of adventitious shoot formation in vitro is an achievement which seems to be within the grasp of a patient investigator. Adventitious shoot formation could be the first step for clonal propagation of apple provided that rooting of shoots, further growth in the field of the produced plantlets and genetic stability are not limiting factors. The immediate purpose of this work was to induce adventitious shoot and/or embryo formation and produce plantlets from them. For this, embryonic explants, i.e. intact embryos, cotyledons and embryo axes were cultured under different combinations of growth regulators in order to assess their morphogenetic capacity. The propagules formed under certain treatments were treated for their further development to plants. Sections were made of explants after short culture in order to trace the origin of the adventitious organs.

Seed of two varieties of apple was used for the induction of morphogenesis in embryonic tissue cultures. The first to which emphasis was given was a Greek variety called Phiriki and the second the widely cultivated and well known variety Golden delicious.

The history of cultivation of the variety Phiriki in Greece is lost with time. Its relatively extended cultivation was very much restricted after the second world war when new varieties mainly for export were introduced and cultivated on a large scale. At the present time the variety Phiriki is mainly cultivated in the region of mount Pelion, county of Magnesia, Greece.

The main characteristics of the var. Phiriki are as follows. It has a very late entry to adult phase. Trees have a long juvenile period and start setting fruits after the 8-10th year of their age. The size of the tree is large. Every year there is heavy fruiting and the shape of the fruits (cylindrical with conical ends) is unusual and they seem to have an extra good storage ability.

A description of this variety is given by Ecomomidis (1959). He and Raptopoulos (1970) in their books mention that there are two variations differing mainly on the time they ripen their fruits. The one is named as early-season Phiriki and the other as late-season Phiriki. The author of this thesis found (unpublished data) that in the area of mount Pelion there are three variations. The two of them in comparison to the third which is described as the main variety differ in physiological characters and show very little and unimportant morphological differences. They ripen their fruits by the end of June beginning of July and the difference between them is the taste of the fruit. The one is sweet and the other sour. The main variety ripens its fruits by the end of September beginning of October. There are no morphological differences among them and obviously the two variations constitute mutations developed from the main variety during its very long cultivation.

Fruits of the var. Phiriki are used fresh, as sun-dried slices (mainly the sour ones) for consumption during the winter and for the production of ouzo. Seeds of Phiriki fruits used in the nurseries for the production of rootstock material produce uniform seedlings which are very vigorous, very resistant to dry environmental

conditions and show very good compatibility with the other commercially grown apple varieties.

The var. Phiriki was selected for this study because its long juvenile phase indicates higher morphogenetic capacity (Murashige, 1974) and because its three mutations could be an important material for genetic manipulations after a successful regeneration study. The var. Golden delicious was selected because it constituted the easiest and cheaper source of seed.

The nutrient medium selected was the Pech et al. (1975) medium. The reasons for choosing this medium were first that it is a modification of the Murashige and Skoog (1962) salt mixture which is the most suitable basic medium for plant regeneration from tissues and callus (Gamborg and Shyluk, 1981) and second because it has been successfully employed in several studies concerning the growth characteristics of apple cell suspensions of fruit parenchyma origin and the metabolism of certain compounds in them (Pech et al., 1975; Ambid and Fallot, 1980; Macheix et al., 1981).

The experimental design for inducing morphogenesis was factorial interaction with two factors, the first a cytokinin and the second an auxin. The cytokinins (BAP and kinetin) were tested at three levels and the auxins (IAA, 2,4-D and NAA) at three, four or five levels. The quantitative expression of morphogenetic results i.e. the percentage of explants which responded to various types of morphogenesis is given in tables drawn in the form of the experimental design. Some of the qualitative results are described in tables drawn in the form of the experimental design, but the majority of them are described in the text and illustrated with photos.

B. MATERIALS AND METHODS

a. Seed collection

Seed of the main var. Phiriki was collected in autumn 1980 and 1981 from a very isolated apple orchard in the area of the village Kalamaki on mount Pelion. Phiriki was the only var. to be cultivated in this orchard which was at least 10 kilometers from the nearest apple orchards in which other varieties were cultivated. This was a strong evidence that the seed collected was produced after self-pollination and excess genetic variability because any cross fertilization had been avoided.

Seed of the var. Golden Delicious was collected only in October from apples purchased from the local market. Seeds were isolated from Golden Delicious apples at a later season because long storage of the fruit affects the physiological status of the seed in respect of dormancy (Villiers, 1975). In this work either dormant or after-ripened seed was used but not any partly after-ripened.

b. Seed sterilization and stratification

Seed was soaked in water overnight and then sterilized with NaOCl. Although 2% available chlorine for 15 min. was enough to surface sterilize the seed, commercial preparation of NaOCl containing 12-14% available chlorine was used for the same time. This high concentration of available chlorine did not damage the seed and because it softened the testa, embryos later could be dissected easily.

Sterilized seeds were rinsed with plenty of sterile water. During rinsing the water obtained a brown colour like the colour of

the testa. This indicated that after sterilization substances contained in the testa and colouring it diffused into the rinsing water. When the rinsing water remained clear the seeds were layered on top of 1% and 3mm thick agar layer in a 9cm Petri dish. Dishes with stratified seeds were sealed with Nescofilm and stored at 4°C in the dark for breakage of dormancy.

If NaOCl with 2% available chlorine was used, the testa remained smooth and hard, and dissection for embryo isolation was difficult.

c. Culture media

The Murashige and Skoog (1962) nutrient medium as modified by Pech et al. (1975) was used for morphogenetic studies with apple embryonic tissues. Stock solutions concentrated 100-1000 times were prepared separately for each substance and stored in glass bottles at 4°C in the dark. Fresh stock solutions were prepared every two months for the inorganic and every month for the organic constituents. Aliquots were taken to prepare the media which were made up to the final volume with single distilled water. Sucrose was added at 3% and the agar used was Difco Bacto agar (Difco Laboratories Ltd., P.O. Box, Central Avenue, East Molesey, KT8 0SE, U.K.). As with carrot, inorganic components were prepared at half the final volume, filter sterilized and their pH was adjusted to 5.7-5.8 prior to filter sterilization.

Ready made media in the form of powder supplied by Flow Laboratories were also used. To these media vitamins according to Pech et al. (1975) were added and growth regulators according to the

treatment. From them the thermolabile ones were filter sterilized and added after autoclaving while the others before autoclaving. The pH was adjusted to 5.7 before autoclaving at 15 psi and 121°C for 15 min.

20ml or 7ml of nutrient medium were poured in a 9cm or 5cm respectively sterile plastic Petri dish forming a 3mm thick layer. After the agar was set, Petri dishes were sealed with Nescofilm and left at room temperature for three days. After this time any contamination could be detected. On the fourth day the dishes were either used or stored at 4°C for a short period not longer than a week before they were used.

d. Embryo isolation

Apple seeds need up to three months chilling temperatures for the removal of their dormancy. After the dormancy is broken the embryo is fully activated physiologically and given proper environmental conditions germination starts. Seeds from the variety Phiriki and Golden Delicious stratified on agar as previously described began germinating after 10 weeks. During stratification the agar layer obtained a brown colour exactly the same as that of the testa. It was concluded that during stratification substances from the testa slowly diffused and accumulated in the agar layer.

After 10 weeks any germinated seeds, i.e. seeds with their radicle just emerged, were discarded and the others were given an extra sterilization with NaOCl, 12-14% available chlorine for 5 min. This additional sterilization contributed to a further softening of the testa which almost completely lost its brown colour and remained

as a thin white layer surrounding the embryo. A rinse with sterile water followed the sterilization of the seed which then was kept in sterile water waiting for the aseptic embryo isolation.

Dormant seeds were soaked in water for 24h and then sterilized for 20 min with NaOCl, 12-14% available chlorine. Then they were rinsed with sterile water and kept soaked overnight. Next day they were similarly treated with NaOCl for 5 min and after rinsing with sterile water they were ready for embryo isolation. After the second sterilization the testa had lost its brown colour at a small area round the pointed end of the seed. Soaking the seed in water and sterilizing it twice resulted in swelling of the seed and softening of the testa. Both facilitated embryo excision.

To isolate the embryos, seed was cut along its peripheral side with care so as to avoid damaging the embryonic tissues. The testa was removed and the embryos were isolated. Viable embryos were of bright white colour with hard tissue. Dead embryos were white with a dark background and with soft rather gelatinous tissues. They were very few and were discarded.

e. Culture of embryonic tissues

The cultures consisted of 1) intact embryos 2) cotyledons and 3) embryo axes. Cotyledons were isolated just by separating them. The embryo axis remained attached to the one of the two separated cotyledons and it could be easily isolated just by touching it with the pointed end of a scalpel or a needle. There were twenty replicates i.e. twenty embryos or cotyledons or embryo axes per treatment distributed in four Petri dishes. 9cm diameter Petri dishes were used for the culture of embryos and cotyledons and 5cm diameter ones for the

culture of embryo axes. Embryos were placed in Petri dishes horizontally lying on one of their cotyledons. Cotyledons were placed with their lower surface in contact with the medium. The lower surface or side of the cotyledon as described is the one which when within the seed is facing the testa or during the seedling development is facing the ground. Embryo axes were placed on the medium horizontally. Petri dishes were sealed with Nescofilm, and incubated at $25 \pm 2^{\circ}\text{C}$ either under continuous light, or dark for a week and then light. The source of light was a common 40 watt bulb fixed at a 10cm height above the incubator and lightening the cultures through a glass window on the top of the incubator. To the light provided by the bulb the diurnal lighting of the room was added. Under these conditions the light intensity was varying from 200 to 1000 lux. The lowest light intensity was prevailing during night while the highest was measured during bright days.

The cultures were observed regularly with a stereoscope. Observations were made under aseptic conditions in a laminar flow cabinet with the Petri dishes open as closed Petri dishes resulted in condensation on the lid preventing observation. Morphogenetic responses of embryos and cotyledons were recorded after 15 days of culture and of embryo axes after a month.

f. Rooting of adventitious shoots

Adventitious shoots were isolated for rooting two months after the cotyledon and three months after the embryo axes cultures were set. They were classified according to their size in three categories each one comprising 1) shoots longer than 2cm 2) shoots

between 1-2cm long and 3) shoots shorter than 1cm.

Isolated shoots were treated in two different ways:

1) they were transferred in 12 x 120mm sterile glass tubes containing 5ml of Pech et al. (1975) medium to which 2mg/l IAA was added. The medium was solidified with 0.8% Difco Bacto agar. Test tubes were plugged with non-absorbent cotton wool, covered with aluminium foil on the top and transferred to an incubator under 16h photoperiod, 1000 lux light provided by warm fluorescent tubes and $25 \pm 1^{\circ}\text{C}$ temperature. 2) They were treated as softwood cuttings. Before planting into the rooting compost shoots were dipped up to 4mm in a solution of 2mg/l IAA and then they were left for some time in a cool place far from any air stream, so as to allow the auxin solution to be absorbed. After that shoots were planted into the rooting compost contained in plastic boxes covered with a polyethylene sheet. Plastic boxes were kept at a room temperature, far from direct light.

For the preparation of the auxin solution the auxin was diluted in half of the final volume of absolute alcohol and was made up to the volume with tap water. Fresh auxin solutions were prepared every time a rooting experiment was set. For the control treatments shoots were treated with a solution containing 50% alcohol and 50% tap water.

Before shoots were treated with IAA solutions or transferred into glass tubes, they were freshly cut just below a node or on the node.

g. Plant microtechnique

Microtechniques are used to study the microscopic details of the structure of plants. In tissue culture they are also used to detect the origin of adventitious organs. In this work the method employed to study the origin of adventitious organs formed in the petiole of cotyledons was the same followed by L. Cowdry (personal communication) for the detection of the origin of organ formation in cereal callus cultures.

Cotyledons were cultured on selected treatments inducing various morphogenetic responses. Six days later, when organ primordia were first visually observed the cotyledons were removed and fixed. To show the existence of buds in the axis of cotyledons seed pre-germinated in agar plates were sown in peat and when 5cm tall they were cut 0.5cm above the PCA. The decapitated seedlings were left for 1 week and then cotyledons together with 0.5cm shoot below the PCA and the remained shoot above the PCA were removed and treated for sectioning. Decapitation of seedlings, i.e. removal of the shoot, stimulated the growth of axillary buds of cotyledons by eliminating the apical dominance. After-ripened embryos having their testa removed i.e. as they were at the time of excision were also fixed for sectioning.

Tissues were killed and preserved in the fluid known as FAA the formula of which is:

Ethyl alcohol	50ml
Glacial acetic acid	5ml
Formalin (40% formaldehyde)	10ml
Water	35ml

This fluid is stable, it has good hardening action and tissues may be stored in it for years. These properties make this formula suitable even for large or impervious objects such as woody twigs, tough herbaceous stems and old roots (Sass, 1958).

Tissues remained in FAA either overnight and then were processed for sectioning or were stored in it to be processed whenever convenient.

h. Dehydration for embedding

Tissues fixed in FAA were dehydrated in alcohol and xylol solutions in the dehydrating series were changed by decanting the liquid from the tissues and promptly flooding the material with a generous volume of the solution next in the series. Changes were made quickly to avoid drying of the tissues because of the volatility of the solutions. The procedure followed is given below:

70% alcohol	1 hr
80% "	" "
95% "	" "
absolute alcohol	" "
absolute alcohol	overnight
25% xylol + 75% absolute alcohol	1 hr
50% xylol + 50% " "	" "
75% xylol + 25% " "	" "
100% xylol	" "

After transfer to xylol containing solutions tissues gradually lost their green colour and finally became transparent.

i. Infiltration and embedding

Infiltration consisted of dissolving the paraplast in the solvent containing the tissues, gradually increasing the concentration of paraplast and decreasing the concentration of solvent. Finally the solvent was eliminated by decantation.

To the xylol solution a few pellets of paraplast were added and after a few hours at room temperature, specimens were transferred to a 35°C incubator. When the paraplast was dissolved a few more pellets were added and the specimen was transferred to a 60°C oven. More pellets were added twice and when paraplast had completely dissolved, the solution of xylol + paraplast was replaced with pure melted paraplast. After 6 hours specimens were transferred in fresh pure melted paraplast and left overnight.

The paraplast matrix in which tissues were embedded served to support the tissues against the fierce stroke of the knife and to hold the parts in proper relation to each other after the sections had been cut. Paper boats of dimensions 3.5 x 2 x 2 cm were constructed. Melted paraplast was poured into the paper boat so as to form a 4mm deep layer. When paraplast was just beginning to set around the sides the specimen was placed in position. Before paraplast was completely solidified more wax was poured in to cover specimen completely and it was allowed to set.

j. Microtome sectioning of material in paraplast

Paraplast around the tissue was trimmed rectangular with the material approximately centered laterally and leaving 1-2mm of paraplast around the specimen. There were two parallel edges so that later a straight ribbon was formed. Tissue was firmly attached to the mounting block and supported, especially on the edge away from the knife by a generous layer of paraplast. Because the specimen was not vertically centred in the paraplast the thicker layer of paraplast was at the top to afford support against the pressure of the cutting action. The thickness of the sections was adjusted to 8 and 10 μ m

k. Affixing sections to the slide

Paraplast sections in the form of a ribbon were stuck to a glass slide with Mayer's Albumen prior to staining. The composition of Mayer's Albumen was:

50ml white of egg

50ml glycerol

1g sodium salicylate

White of egg was shaken for 5 min in a mixer with a few drops of acetic acid. The other ingredients were added and shaking for another 10 min. followed. The mixture was then filtered into a clean bottle with the help of a vacuum pump. The so prepared Mayer's Albumen was diluted. 10 drops of it were taken in 20ml water and 1-2ml of it were transferred to each slide. New slides were used and they were cleaned shortly before using. The slides were placed on a 45°C heater and left overnight to dry.

1. Staining sections

Sections affixed to slides were stained and processed by immersion in reagents in staining jars. Each jar contained enough reagent to cover the slides completely. The method of Jane O'Hara (personal communication) was followed for staining. The solutions and the time of immersion in each of them are given below:

xylol	5 min
50% xylol + 50% Absolute ethanol	" "
95% Ethanol	3 min
70% " "	" "
50% "	" "
30% "	" "
0.05% Toluidine Blue (aqueous solution)	15 min
30% Ethanol	3 min
50% "	" "
70% "	" "
95% "	" "
Absolute Ethanol	" "
Xylol	5 min

After the affixed sections had been dried on a heater, the paraplast was dissolved by immersing the slides in the first xylol. Slides after staining were placed with tissue upward on a sheet of dry blotting paper. The whole work was done rapidly to avoid drying of the tissues. Excess xylol was removed, a drop of Canada balsam was put on the tissues, and a clean coverslip was lowered obliquely onto the Canada balsam.

C. RESULTS

a. INTERACTION OF IAA-BAP

In one experiment to study the interaction of IAA and BAP, IAA and BAP were used at concentrations 0, 0.2, 2 and 0, 0.1, 1mg/l respectively, while in the other experiments the BAP was used at concentrations 0, 0.2 and 2mg/l. These concentrations of BAP were used instead of the previous because in a separate experiment (Table 5) with cotyledons in which only BAP was used the concentration of 2mg/l BAP gave the best morphogenetic response. All these experiments were carried out under continuous light.

a-1. Embryo culture

Embryos were cultured on media containing the above-mentioned concentrations of IAA-BAP and combinations of them. From the first day of their culture the cotyledons started moving apart and from the third day and on they had, in relation to the embryo axis, the normal position they have in growing seedlings. On the second day cotyledons started forming chlorophyll and expanding while the growth of the radicle and the plumule was obvious.

After 15 days of culture excised embryos under different treatments responded in the following way: on media containing no growth regulators (control) they developed towards normal plants having a shoot and a root with a length ranging from 0.5-4cm and 2-5cm respectively. Along the primary root secondary roots had been developed. Similar was the development of embryos grown on media containing 0.1 and 0.2mg/l BAP. The range of shoot and root length was 0.5-3cm and 3-7cm respectively. Secondary roots were formed along the primary root. The hypocotyl grew 2-2.5cm long and 1.5mm

thick (diameter) and it was chlorophyllous with the control treatment as well as ^{with} 0.1 and 0.2mg/l BAP.

Embryos responded very characteristically when cultured on media containing 1 and 2mg/l BAP. The plumule developed to a shoot 1-3cm long, the lateral buds of which including the axillary buds of cotyledons, sprouted forming a rosette-like shoot system. The root growth compared to the previous treatments was inhibited. With 1mg/l BAP 50% of embryos had a root 2-10mm long and the other 50% had a range of 1-4cm. With 2mg/l BAP all the embryos had a root length varying from 2 to 15mm. The hypocotyl grew shorter, 1-2cm long, and thicker, 3mm in diameter, it was chlorophyllous and along its surface adventitious buds were formed. Adventitious buds were also formed on the base of the petiole of the cotyledons.

IAA treatments had a different effect. With 0.2mg/l all the embryos developed a shoot 2-4cm long. The root and the hypocotyl developed normally but in some embryos between the chlorophyllous hypocotyl and the white root a white callus developed from which adventitious roots were arising. 2mg/l IAA had a more striking effect. All the embryos developed a normal shoot 0.4-4cm long and in all of them the hypocotyl together with the root developed to a white callus mass. In most of these calluses adventitious roots were formed. Roots were also formed directly from cotyledons without any intervening callus (Fig. 1).

Interaction of IAA/BAP at concentrations 0.2/0.1 and 0.2/0.2mg/l respectively resulted mainly in formation of adventitious roots arising from callus formed between the hypocotyl and the root.



Fig. 1: Apple embryo explants of the var. Phiriki cultured on Pech et al. (1975) medium containing 2mg/l IAA. a: The root and the hypocotyl have grown to a callus mass from which adventitious roots arise (x 1). b: Adventitious roots arise directly from the green cotyledons (x 1.2).

In one replicate of the treatment 0.2/0.1 mg/l adventitious roots arose from the petiole of the cotyledon and the base of the shoot meristem, while in one replicate of the treatment of 0.2/0.2mg/l, apart from the adventitious roots, adventitious buds were formed on the hypocotyl. Under the 0.2/1mg/l treatment embryos formed adventitious roots from callus developed between the hypocotyl and root, or adventitious buds on the hypocotyl and the lower surface of the cotyledons. The same morphogenetic reaction plus the simultaneous formation of adventitious roots and buds was observed under the treatment 0.2/2mg/l.

The formation of structures which were neither adventitious buds nor embryos was noticed in the lower surface of the cotyledons of one embryo in the treatment 0.2/1mg/l. These structures which did not develop further were described as foliar structures (Fig. 2).

The response of embryos cultured on combinations of IAA/BAP at concentrations 2/0.1 and 2/0.2mg/l was similar to that of only 2mg/l IAA. The hypocotyl and the root grew to a callus from which adventitious roots were arising. Roots were also arising from cotyledons without any intervening callus. Only in one embryo under the treatment 2/0.2mg/l apart from the adventitious roots, adventitious buds were also formed on the hypocotyl. Combination of 2/1mg/l favoured root formation while under the combination 2/2mg/l root, bud and formation of both occurred. In all the interaction treatments the shoot grew normally except in the treatment 2/2mg/l where shoots were formed from lateral buds including the axillary buds of cotyledons. Under all treatments of this experiment cotyledons



Fig. 2: Foliar structures formed on the lower surface of cotyledon explants isolated from apple embryos of the var. Phiriki and cultured on Pech et al. (1975) medium supplemented with 0.2mg/l IAA and 1mg/l BAP (x 15).

became dark green and expanded. Their dimensions (average) were 12 x 9mm in comparison to 6.1 x 4.0mm at the time the culture was set. That means that cotyledons increased their surface by a factor of approximately 4.5 after fifteen days.

Summarising, BAP at concentrations 1 and 2mg/l induced bud formation on the hypocotyl and lower surface of the cotyledon, favoured shoot development from lateral buds, inhibited root growth at 2mg/l and caused characteristic growth of the hypocotyl (Fig. 3). On the contrary 0.1 or 0.2mg/l BAP favoured root growth of cultured embryos. IAA at 0.2mg/l induced root formation from callus formed between the hypocotyl and the root. IAA at 2mg/l induced root formation first from callus developed from the hypocotyl and the root and second directly from cotyledons. It should be emphasized here that under this treatment the part of the embryo above the point cotyledons arise (PCA) including the cotyledons continued its growth towards a normal plant and it is only the part below the PCA affected by the treatment. The types of morphogenesis and the percentages of embryos responded to them are shown in Table 1.

a-2. Cotyledon culture

a-2.1. Intact cotyledons

Isolated cotyledons started forming chlorophyll from the first day of their culture. On the third day they were dark green and expansion was obvious. After a few days the formation of a petiole having lighter green-white colour was distinct. A very distinct petiole bearing bracts was also formed in cotyledons of growing seedlings (Fig. 4). Morphogenesis occurred under all treatments except

Table 1

Morphogenetic responses in explants isolated from after-ripened embryos of apple var. Phiriki and cultured on Pech et al. (1975) medium containing IAA, BAP and combinations of them. Three values are given for each treatment. The first from the left is the percentage of explants which formed adventitious roots, the second buds and the third buds and roots.

Explant	Embryo			Embryo axis		
	IAA			IAA		
	0	0.2	2	0	0.2	2
Concentration mg/l	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
BAP	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0
2	0	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	0	0	0

Observations were recorded after 15 days for embryo and after 30 days for embryo axis explants.

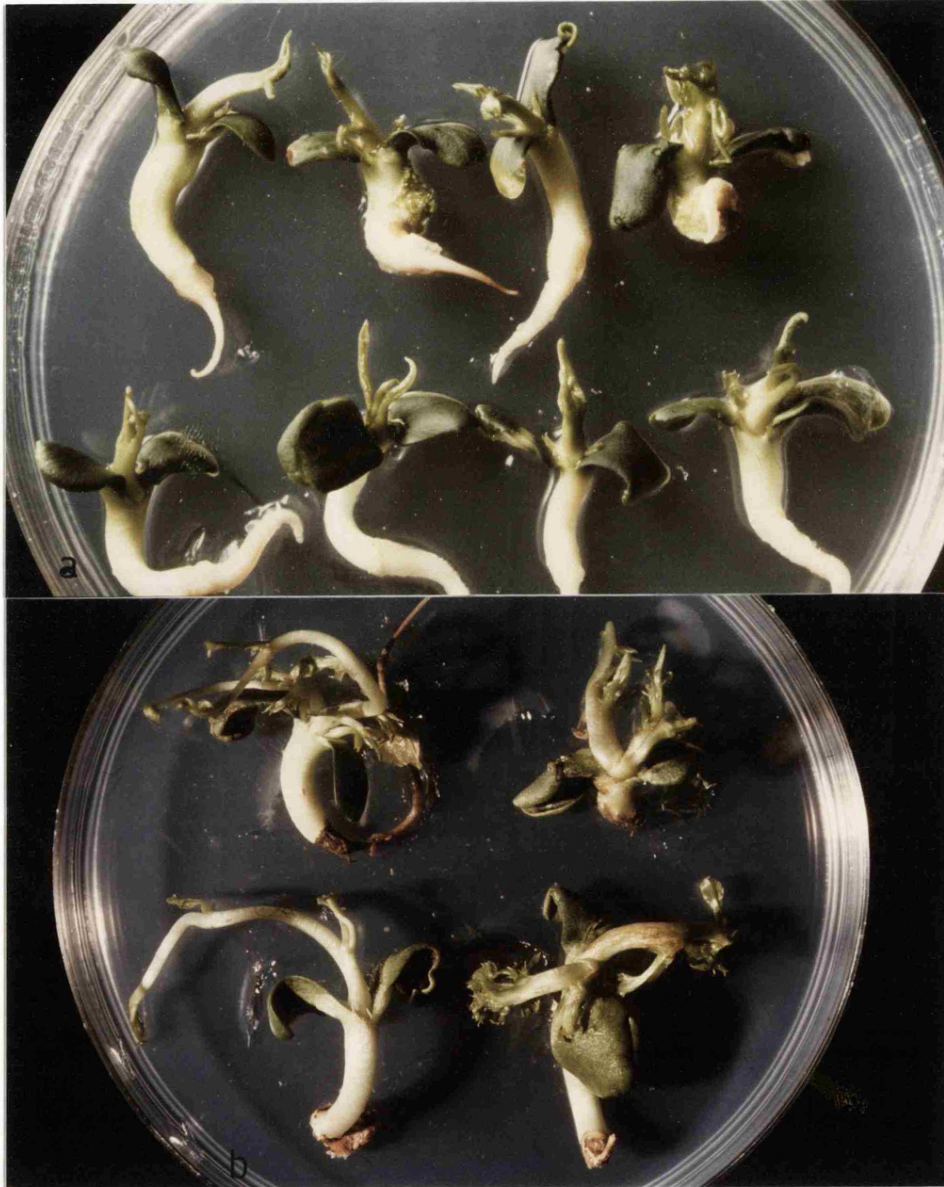


Fig. 3: Apple embryo explants of the var. Phiriki cultured on Pech et al. (1975) medium supplemented with 1mg/l (a) and 2mg/l BAP (b).

a: Lateral shoots arising from the cotyledonary buds, characteristic growth of the hypocotyl and inhibition of the root growth.

Adventitious buds can be seen on the hypocotyl of the second from the left embryo on top (x 1.4). b: Lateral shoots formed under the BAP effect, characteristic growth of the hypocotyl and inhibition of root growth (x 1.2).



Fig. 4: a: Cotyledon and lower halves of cotyledon explants isolated from apple embryos of the var. Phiriki after fifteen days culture on Pech et al. (1975) medium containing no growth regulators develop a very distinct petiole (x 1). b: Cotyledons isolated from growing seedlings show a distinct petiole too (x 7).

the control, 0.1 and 0.2mg/l BAP.

Morphogenetic responses were similar to those of cultured embryos. BAP induced adventitious bud formation. These buds could visually be observed after six days of culture. They continued their growth and shoots developed. Optimum concentration for the induction of bud formation was found to be 2mg/l (Tables 2 and 3).

The main characteristics of the BAP treatments were 1) that adventitious bud formation was strictly localized at the petiole 2) the complete lack of callus and the origin of buds directly from the petiole and 3) that in contrast with adventitious buds developed on the hypocotyl of cultured embryos, buds developed on the petiole of cultured cotyledons continued their development to shoots (Fig. 5). On media containing 4mg/l BAP inhibition of chlorophyll formation was observed. The cotyledons had a yellow or yellow green colour.

IAA induced adventitious root formation. As with buds, root formation occurred within a week and was also localized at the petiole, which in some cases was very little developed and roots seemed to arise from the base of the cotyledon. The percentage of rooted cotyledons was high under 0.2mg/l and higher with 2mg/l. From each cotyledon there were several roots arising. They were long, showing very little secondary root formation (Fig. 6a). With 2mg/l IAA 40% of the cotyledons formed also a callus mass. Callus and roots were formed at the same area (petiole) but their formation was independent. Sometimes root formation preceded callus formation but in most cases callus was formed before roots.

Interaction of IAA/BAP at concentrations 0.2/0.1 and 2/

Table 2

Morphogenetic responses in cotyledon explants isolated from after-ripened embryos of apple var. Phiriki and cultured for 15 days on Pech et al. (1975) medium containing IAA, BAP and combinations of them. Three values are given for each treatment. The first from the left is the percentage of explants which formed adventitious roots, the second buds and the third buds and roots.

Explant Concentration mg/l	Cotyledon			Cotyledons cut at their base					
	IAA			IAA					
	0	0.2	2	0	0.2	2	0	0.2	2
0	0 0 0	65 0 0	95 0 0	0 0 0	5 0 0	15 0 0	0 0 0	0 0 0	0 0 0
0.2	0 0 0	70 0 5	80 0 10	0 0 0	5 0 0	15 0 0	0 0 0	0 0 0	0 0 0
2	0 100 0	5 80 15	20 0 65	0 25 0	0 35 0	15 20 20	0 0 0	0 0 0	0 0 0

roots buds both

Table 3

Adventitious shoot formation in cotyledon explants isolated from after-ripened embryos of apple var. Phiriki and cultured^{*} for 20 days on Pech et al. (1975) medium supplemented with BAP or kinetin.

Concentration	% of explants formed adventitious buds with BAP	% of explants formed adventitious buds with kinetin
0	0	0
0.2	0	0
1	40	5
2	100	20
4	55	10

* The experiment was carried out under dark for a week and then continuous light.

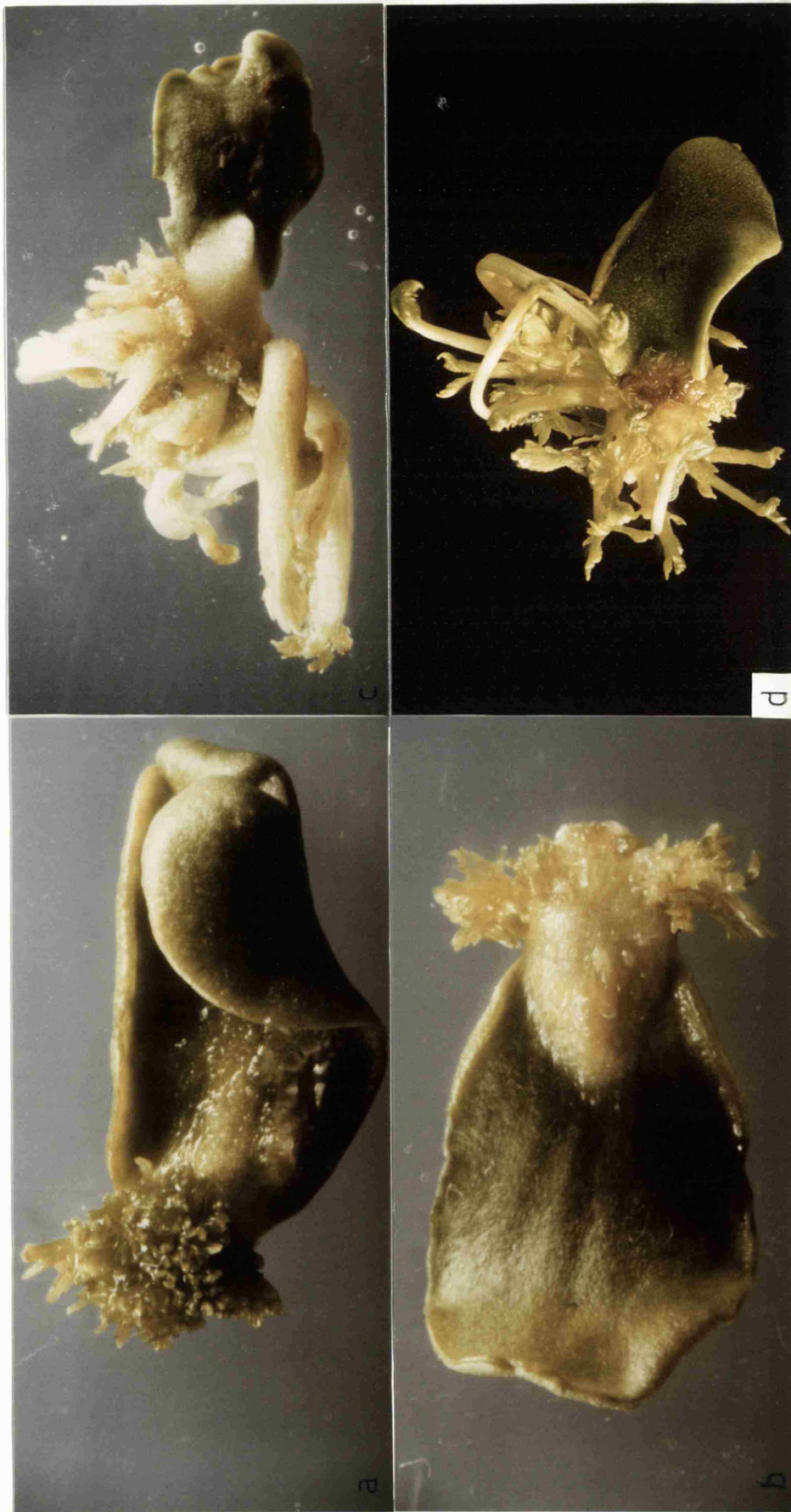


Fig. 5: Adventitious shoot formation on cotyledon explants isolated from apple embryos of the var. Phiriki and cultured on Pech et al. (1975) medium supplemented with 2mg/1 BAP. a, b and c are showing the view from the lower surface and d is showing the view from the upper surface of the cotyledon. Note the complete lack of callus, the origin of the adventitious shoots from the petiole (b-c), the small laminae and the long internodes of the adventitious shoots (c-d) (x 5, x 5, x 2, x 2).



Fig. 6: Adventitious root and bud formation on cotyledon explant isolated from apple embryo of the var. Phiriki and cultured on Pech et al. (1975) medium supplemented with 2mg/l IAA and 2mg/l BAP. Roots are mainly formed along the cut side and lower surface of the cotyledon. Buds are seen in the form of clusters on the upper surface of the cotyledon (x 15).

0.1mg/l gave the same results as with IAA treatments. The difference was that usually one big root having many secondary roots formed on each cotyledon (Fig. 6b). When IAA/BAP were combined at concentrations 0.2/0.2 and 2/0.2mg/l cotyledons responded morphogenetically in the same way, plus that in a small percentage both roots and buds were formed on the petiole. Combination of IAA/BAP at concentrations 0.2/2 and 2/2mg/l resulted mainly in formation of roots and buds and secondarily in formation of only roots or only buds. When concentrations of 0.2/1 and 2/1mg/l were used roots or buds were formed but formation of both did not occur as expected and the experiment was not repeated.

Whenever formation of both roots and buds occurred in the same cotyledon, roots were always formed first and the appearance of buds always followed root formation. Under only BAP or IAA treatment buds and roots respectively started appearing at about the same time, i.e. 6 days after the culture was set. Organ formation was again localized at the petiole and the impression was given that roots were usually formed along the dorsal side and the lower surface of the petiole while buds were formed on the upper and lower surface (Fig. 7). Buds formed in combinations of IAA/BAP did not develop further to shoots in contrast to those formed under the 2mg/l BAP treatment.

As mentioned previously expansion of cotyledons was obvious from the third day of their culture. Roots or buds formed at the petiole could be seen some time from the fourth day but usually the majority of them from the sixth day and on. This observation showed that morphogenetic processes and morphogenesis were



Fig. 7: Adventitious root formation on cotyledon explants isolated from apple embryos of the var. Phiriki. a: When cultured on Pech et al. (1975) medium supplemented with 2mg/l IAA and b: when cultured on the same medium supplemented with 2mg/l IAA plus 0.1mg/l BAP. Note the formation of secondary roots in the presence of BAP (x 1.3, x 1).

occurring while cotyledon was expanding.

For an assessment of the increase (expansion) of the cotyledon two dimensions were measured. The one described as length was the distance from the edge of the petiole to the opposite end of the cotyledon and the other described as width was the longest distance between two opposite points of lateral sides. The two dimensions were multiplied and the outcome was divided by the size of cotyledons at the time of excision to give the increase of the size of cultured cotyledons. The average cotyledon size at the time of excision was similarly assessed by measuring two hundred cotyledons. The average was found to be 24.4mm^2 (average dimensions 6.1-4.0mm). Table 4 shows the increase of the size of cotyledons after 30 days of culture.

a-2.2 Cotyledons cut at their base

Adventitious bud formation could not be confused with the existence of the axillary bud because the first were formed at high numbers on every side of the petiole. Separate experiments were set in which the axillary bud of the cotyledon had been removed. For this isolated cotyledons were cut 1mm deeper from the inner point of their base as shown in (Fig. 8). The area removed was that one which was determined to form later the petiole. This was confirmed with separate experiments in which cotyledons cut at depths of 0, 0.5, 1, 1.5 and 2mm from the inner point of their base were cultured in control media. Cotyledons cut deeper than 1mm did not develop on culture any tissue similar to that of the petiole. When they were cut 1mm deep, tissue similar to that of the petiole developed occasionally and this was probably because of the variation of the size of the cotyledon. All the cotyledons cut 0.5mm deep developed some petiole

Table 4

Relevant increase of the size of cotyledon explants isolated from after-ripened embryos of apple var. Phiriki and cultured for 30 days under continuous light on Pech et al. (1975) medium supplemented with IAA, 2,4-D, and combinations of them with BAP.

Concen- tration mg/l	IAA			2,4-D			
	0	0.2	2	0.1	1	2	4
BAP	0	2.89	3.38	3.58	2.89	2.72	2.74
	0.1	3.45	4.38	4.29	3.27	3.11	3.02
	1	3.91	3.83	4.98	3.43	2.99	3.12

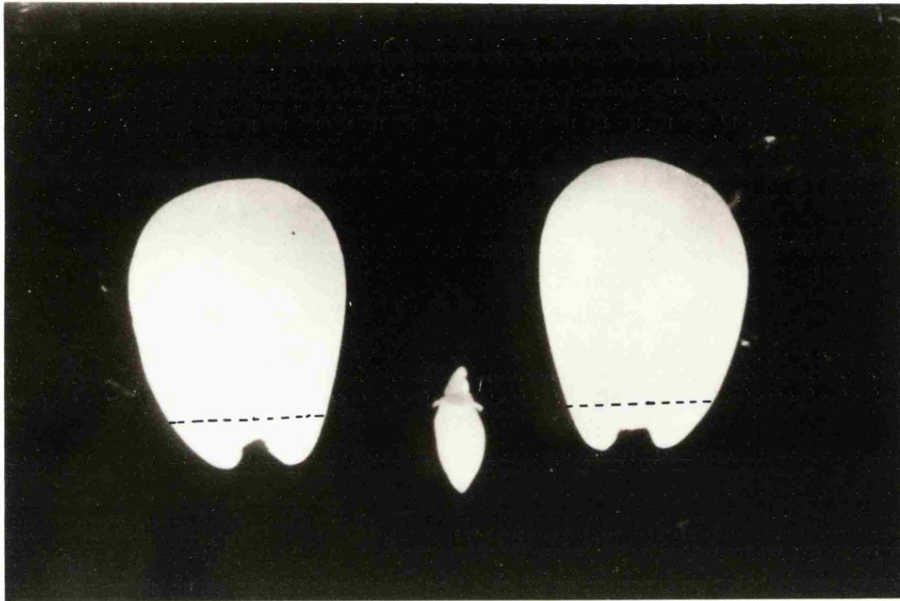


Fig. 8: Cotyledon and embryo axis explants isolated from an apple embryo of the var. Phiriki at the time of excision. The area of cotyledon below the dotted line is that one which is determined to form the petiole (x 6.5).

tissue, while those which were cultured intact developed a normal petiole.

These experiments together with the others in which halves of cotyledons were cultured (Chapter 4C,e) provided information on which part of the cotyledonary tissue is responsive to morphogenesis. They also indicated that the existence of the bud did not play any role in the induction of organ formation which was induced mainly because of the treatment.

Cotyledons cut at their base gave similar morphogenetic responses to intact. The differences were that the percentage of the morphogenetically responded cotyledons per treatment was much lower (Table 2), the number of roots and buds formed in each cotyledon was smaller and their growth and development was very poor. Roots remained short (up to 1cm) and thin and buds did not develop further to shoots. Their formation could be visually observed after 15-20 days of culture which is three times longer in comparison to the time they could be observed in intact cotyledons.

a-3. Embryo axis culture

After three days culture shoot and root development and elongation of the hypocotyl were obvious. Shoot and hypocotyl had already developed chlorophyll and under certain treatments growth towards callus started. After 30 days the response of embryo axes on each treatment was as following. On control media and media containing 0.1 or 0.2mg/l BAP embryo axes developed a 5mm root, a 4mm hypocotyl and in some of them from the shoot meristem very small green leaves were formed which did not develop further. Under

2mg/l BAP adventitious buds were formed on the upper part of the hypocotyl which grew shorter and thicker in comparison to control. The shoot meristems developed further and shoots arose from the apical and lateral buds. Embryo axes grew characteristically when cultured on a medium containing 4mg/l BAP. All the buds, apical and lateral, sprouted and formed a rosette consisting of many shoots. The hypocotyl grew 4-5mm long and 3mm thick and had the form of a barrel. Root growth was very little and in some axes the root grew into callus. Adventitious bud formation was observed on the upper part of the hypocotyl.

On media containing 0.2mg/l IAA axes developed towards little plants. Their development was better in comparison with the control treatment but still small. They had a shoot about 1cm, hypocotyl 0.5cm and root 2cm long. In some of them callus was formed between the hypocotyl and the root and from this callus adventitious roots were arising. With 2mg/l IAA in most of the embryo axes the hypocotyl together with the root developed to a callus from which many adventitious roots were formed. It was characteristic that roots were arising from the area of callus which was below the shoot meristem, which developed to a small shoot 0.5-1cm long. Some embryo axes grew to a white or brown callus with no morphogenetic response.

Combinations of IAA/BAP at concentrations 0.2/0.1 and 0.2/0.2mg/l resulted mainly in development of the embryo axes towards normal plants. The shoot was about 1cm, the hypocotyl 0.6cm and the root 4cm long. Under this treatment the best development of embryo axes towards normal plantlets was observed. A number of axes formed a callus between the hypocotyl and the root. From some of these

calluses adventitious roots were formed. Only in one axis under the treatment 0.2/0.2mg/l roots developed from callus formed between the hypocotyl and the root and buds formed on the hypocotyl. Combinations at concentrations 0.2/1 and 0.2/2mg/l resulted in inhibition of root growth in comparison to the previous treatment. The root grew 1cm long while the shoot and the hypocotyl grew 1 and 0.5cm long respectively. Adventitious roots were formed on callus developed between the hypocotyl and the root, and adventitious buds were formed on the upper part of the hypocotyl. When IAA was combined with BAP at concentrations 2/0.1 and 2/0.2mg/l the shoot developed to 0.5cm long, the hypocotyl either as a whole or except a small area just below the PCA developed to a white callus from which adventitious roots were arising and the root grew 4cm long. Under the treatment 2/0.2mg/l in a small number of embryo axes adventitious bud formation took place together with root formation. Buds were formed on the upper area of the hypocotyl which did not grow to callus. Simultaneous bud and root formation happened mainly under the 2/2mg/l IAA/BAP treatment. Roots were formed on callus developed from the hypocotyl and the root. Buds were formed on a small area of the upper part of the hypocotyl which like in the previous treatment remained green and did not participate in callus formation. In some axes only root or only shoot formation was observed.

Root formation started 10-15 days after the culture was set. Buds appeared after a month and they continued their development to shoots only under BAP treatments. Under any combination of IAA/BAP buds did not develop further. In some cases it seemed that adventitious buds were formed in the points of the embryo axis where coty-

ledons arise. The general tendency for buds was to develop on the upper part of the hypocotyl and close to the plumule. The various types of morphogenesis and the percentage of embryo axes which responded are shown in Table 1.

a-4. Transfer from morphogenetic media to media permissive of organ development

Although bud and root formation was induced on embryonic tissues cultured on media containing IAA, BAP and combinations of them, in many cases buds did not develop further to shoots. With cultured cotyledons and embryo axes adventitious buds continued their growth only under BAP treatments while with embryos, buds did not develop at all under any treatment. Adventitious roots developed normally under any treatment.

To see if buds could grow more and develop to shoots, cultured embryos, cotyledons and embryo axes from every treatment were transferred to media containing the growth regulators at a reduced concentration. IAA, BAP and combinations of them were used at concentrations of 0, 0.2-0, 0.1, 0.2mg/l respectively. Embryos and cotyledons were transferred after 15 days and embryo axes after one month culture on the first medium. From the twenty replicates 16 were transferred (4 to each of the four media) and 4 remained on the first medium.

The percentages of cotyledon and embryo axis explants giving morphogenetic responses before and after the transfer are shown in Table 5. After the transfer the percentage of morphogenetically responding explants was higher. This was because initiation of organ

Table 5

Morphogenetic responses in explants isolated from after-ripened embryos of apple var. Phiriki and cultured on Pech et al. (1975) medium containing IAA, BAP and combinations of them. Three values are given for each treatment. The first from the left is the percentage of explants which formed adventitious roots, the second buds and the third buds and roots.

Explant *		Cotyledon			Embryo axis			3 explants which respond morphogenet- ically before and after transfer									
Concentration mg/l		IAA			IAA												
		0	0.2	2	0	0.2	2										
0	BAP	5	0	0	25	0	0	30	0	0	0	45	0	0	Before		
		0	0	0	35	0	0	45	0	0	0	20	0	0	After		
		5	0	0	60	0	0	75	0	0	0	65	0	0	Total		
0.2		0	0	0	35	0	5	25	0	5	0	0	0	0	Before		
		0	0	0	10	0	0	35	0	0	0	25	0	0	After		
		0	0	0	45	0	5	60	0	5	0	25	0	0	Total		
2		0	40	0	5	20	15	10	15	35	0	15	0	10	5	10	Before
		0	30	0	0	10	5	25	0	5	0	0	0	0	0	5	After
		0	70	0	5	30	20	35	15	40	0	15	0	10	5	15	Total

* Observations were recorded after 15 days for cotyledon and after 30 days for embryo axis explants.

formation had been induced on the first medium and organs appeared after the transfer to the second medium. Organs also appeared after the fifteenth day on explants which remained on the first medium and were not transferred (controls). Under all the treatments used in these experiments (45 in total) buds did not show any difference in their development in comparison to those formed when explants were not transferred to media containing low concentrations of growth regulators. Buds and shoots already developed on cotyledons, and embryo axes cultured on media containing BAP, continued their growth after transfer. Buds formed on the hypocotyl and cotyledons of cultured embryos under all treatments, and buds formed on cotyledons and embryo axes cultured on media containing combinations of IAA/BAP did not develop further after transfer, in contrast to roots which continued their normal growth under every treatment.

b. INTERACTION OF 2,4-D and BAP

In the first experiment 2,4-D and BAP were combined at concentrations 0, 0.1, 1, 2 and 4mg/l and 0, 0.1, 1mg/l respectively. In other experiments BAP was used at concentrations 0, 0.2 and 2mg/l because as already mentioned earlier BAP gave best morphogenetic response at 2mg/l. All these experiments were carried out under continuous light or dark for a week and then continuous light.

b-1. Embryo culture

During the first three days embryos cultured under continuous light on the above-mentioned concentrations of 2,4-D, BAP and combinations of them responded in a way similar to that described in a-1. Similar was also the response of embryos cultured on control media and media containing 0.1, 1 and 2mg/l BAP. Morphogenetic

responses recorded after 15 days of culture on media containing 2,4-D were different in comparison to those observed after culture on media containing IAA.

Under 0.1mg/l 2,4-D embryos developed towards normal plants having a distinct shoot, hypocotyl, root and well developed green cotyledons. The length of shoots and roots was varying from 1-8cm and the hypocotyl was 1.5-2cm long. Secondary root formation was observed along the primary root of some embryos. With 0.2mg/l 2,4-D embryos responded in a similar way. The differences were that shoots grew shorter up to 2cm and with some embryos the root grew into callus.

With 1, 2 and 4mg/l 2,4-D the root and the hypocotyl of the embryos formed a big rather spherical white callus mass. Green cotyledons seemed to arise from that callus and shoots or apical buds seemed to be sitting on the callus (Fig. 9). Shoot meristems usually developed to a shoot up to 0.5cm long under the concentrations of 1mg/l 2,4-D but remained as an apical bud under the higher concentrations of 2,4-D. It should be mentioned here that these treatments of 2,4-D affected mainly the part of the embryo below PCA while cotyledons developed normally and buds retained their normal shape but their development was inhibited. These 2,4-D treatments had an effect similar to 2mg/l. On the upper surface of the petiole of the cotyledons white globular structures were formed which later were found to be proembryos (Fig. 10). These proembryos could visually be observed after one week of culture. Although sometimes callus grew over the proembryos, and in certain cases the lower part of the petiole seemed to participate in callus formation, the proembryo formation was independent and could not be related to callus formation. This was



Fig. 9: Apple embryo explants of the var. Phiriki cultured on Pech et al. (1975) medium supplemented with 2mg/l 2,4-D. The cotyledons grew normally, the root and the hypocotyl grew to a callus and the growth of the apical bud has been inhibited (x 1).

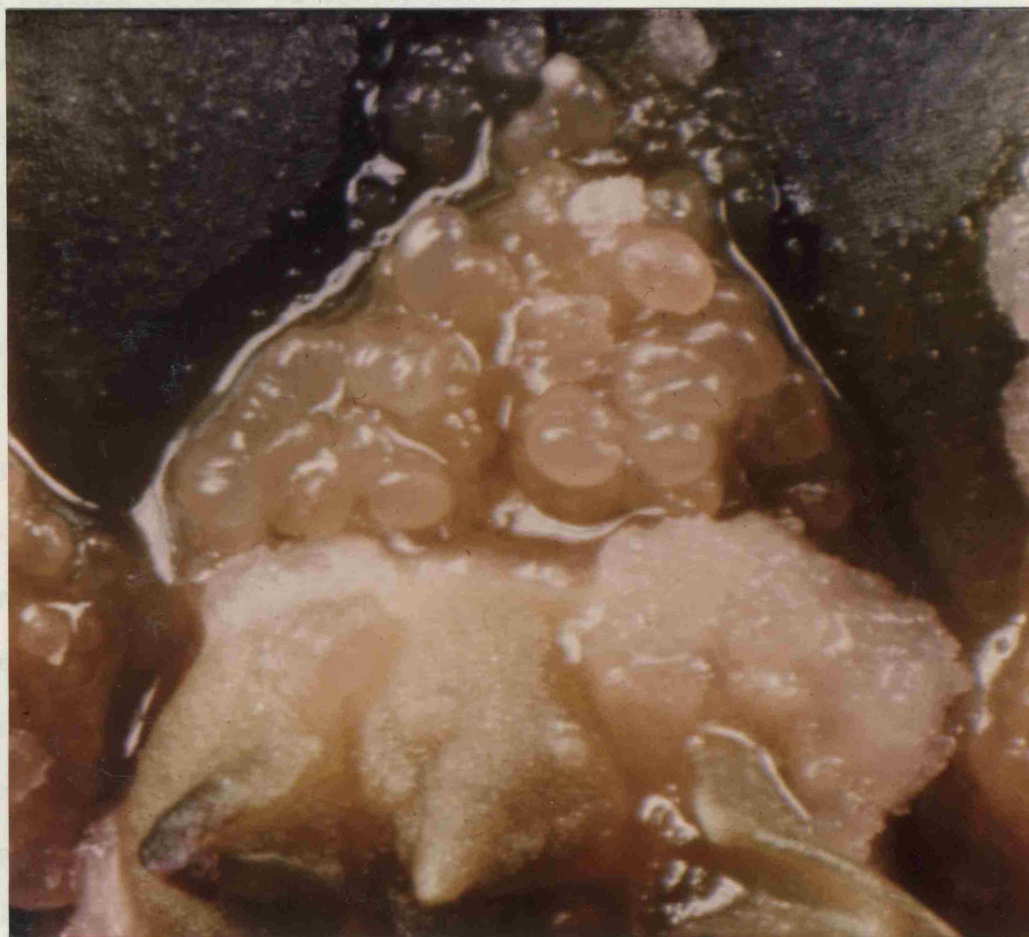


Fig. 10: Proembryos formed at the petiole of the cotyledon of apple embryo explants var. Phiriki when cultured on Pech et al. (1975) medium supplemented with 4mg/l 2,4-D (x 17).

confirmed by the fact that a few times proembryos were formed along the surface or edge of the cotyledon where no callus was formed. Proembryos formed under all treatments either proliferated and fused together into a callus or turned to brown colour without developing further (Fig. 13).

Embryos cultured in the same concentrations of 2,4-D i.e. 0.1, 1, 2 and 4mg/l combined with 0.1 or 0.2mg/l BAP responded in the same way. Callus was formed from the root and the hypocotyl and proembryos were formed on the upper surface of the petiole. The only difference was that with 0.1 and 1mg/l 2,4-D combined with BAP the shoots grew slightly longer while with higher concentrations they had again the form of a big apical bud with two or four of the surrounding protective leaves expanded. When the previous concentrations of 2,4-D were combined with 1 or 2mg/l BAP the embryos responded in the following way. Under the combination of 0.1/1-0.1/2mg/l their hypocotyls grew 2cm long on average and 3mm in diameter, the shoot was 2cm long and had the form of a rosette because lateral buds including the cotyledonary sprouted and many shoots were formed. Adventitious bud formation was observed on the petiole of the cotyledons and on the hypocotyl. In combinations of low 2,4-D concentrations, 0.1 or 0.2mg/l with high concentration of BAP, 2mg/l, "tearing" of the root of the embryos occurred because of the tendency of the root tissue to form a very friable callus (Fig. 11).

Under the combinations 1/1 and 1/2mg/l 2,4-D/BAP the shoot meristem developed to a big apical bud from which a shoot 0.5cm long developed. At the same time lateral buds including the cotyledonary sprouted and formed a rosette-like shoot system. The hypocotyl grew



Fig. 11: Apple embryo explants of the var. Phiriki cultured on Pech et al. (1975) medium supplemented with 0.2mg/l 2,4-D and 2mg/l BAP. Note the tendency of the root to form callus (x 1).

1cm long and 4mm in diameter and the root grew into callus. On the petiole of the cotyledons many globular structures of white colour were formed. These structures were considered as pro-embryos and not as adventitious buds. Similar was the response of cultured embryos on media containing combinations of 2,4-D/BAP at concentrations 2/1, 2/2, 4/1 and 4/2mg/l.

These results were obtained from two experiments. In the first experiment 2,4-D and BAP were combined at concentrations of 0, 0.1, 1, 2, 4 and 0, 0.1, 1mg/l respectively and the cultures were under continuous light. In the second experiment 2,4-D and BAP were combined at concentrations of 0, 0.1, 1, 2, 4 and 0, 0.2, 2mg/l respectively. The cultures were set under dark for a week and then under continuous light. When the cultures were exposed to light after a week it was observed that the embryos developed like the ones cultured under continuous light. The difference was that they were etiolated although the cotyledons had a yellow-greenish colour which indicated chlorophyll formation. The idea of setting the cultures under dark for a week was that greening, which means further differentiation, might have an inhibitory effect on the formation of adventitious buds and proembryos and their development. Immediately after the exposure to light the embryos formed chlorophyll and continued their normal growth and development. Table 6 shows the percentage of embryos which formed either adventitious buds or proembryos under one week dark and then continuous light.

Table 6

Morphogenetic responses in after-ripened embryo explants of apple var. Phiriki cultured for 15 days on Pech et al. (1975) medium containing 2,4-D, BAP and combinations of them under one week dark and then continuous light. Two values are given for each treatment. The first from the left is the percentage of explants which formed adventitious buds and the second of explants which formed proembryos.

Concentration mg/l		2,4-D					
		0		0.2		1	
BAP	0	0	0	0	0	0	70
	0.2	0	0	0	15	0	85
	2	55	0	10	0	0*	100

* Bud primordia were formed but they could not be visually observed.

b-2. Cotyledon culture

b-2.1. Intact cotyledons

Cotyledons were cultured under two combinations of 2,4-D/BAP. The first combination was 0, 0.1, 1, 2, 4/0, 0.1, 1 and the second was 0, 0.2, 2, 4, 8/0, 0.2, 2mg/1 respectively. Under continuous light chlorophyll formation started from the first day of their culture and on the third day cotyledons were dark green and expanded. Morphogenetic responses were similar to those of cultured embryos and occurred in all treatments except 0, 0.1 and 0.2mg/1 BAP. The effect of BAP itself is described previously in a-1.

Under 0.1mg/1 2,4-D adventitious roots were formed. With 0.2mg/1 root formation was rather occasional. With higher 2,4-D concentrations proembryos were formed on the petiole of each cotyledon together with callus arising from the edge of the petiole. Big callus (Fig. 12) was formed under the concentrations of 2 and 4mg/1 2,4-D. In these treatments sometimes callus was overgrown and the proembryos were hidden underneath. Adventitious root formation was observed when 2,4-D/BAP were combined at concentrations 0.1/0.1 and 0.2/0.2mg/1. The combinations of 0.1/1 and 0.2/2mg/1 resulted in adventitious bud formation. In one cotyledon of the treatment 0.1/1mg/1 adventitious roots, buds and foliar structures were formed simultaneously. The main characteristic of these two treatments was the complete lack of callus.

When concentrations of 1, 2, 4 and 8mg/1 of 2,4-D were combined with 0.1 or 0.2mg/1 BAP cotyledons formed proembryos on the petiole and callus. Proembryos and callus were also formed when the previous concentrations of 2,4-D were combined with 1 or



Fig. 12: Callus formation in cotyledon explants isolated from apple embryos of the var. Phiriki after fifteen days culture on Pech et al. (1975) medium supplemented with 2mg/l (two cotyledons from left) and 4mg/l 2,4-D (the other two cotyledons)(x 1).

2mg/l, 2,4-D. In these combinations on the petiole of the cotyledons and among the white globular proembryos some small green structures were observed. These structures which did not develop further were very similar to adventitious buds developed under BAP treatments and they were considered as such.

As with adventitious buds proembryos under all treatments were mainly formed on the petiole of the cotyledon and could visually be observed after one week culture. Their formation though was not so strictly localized as with adventitious buds. Callus formation was independent and could not be related to proembryo formation which were formed directly from the petiole tissue. Proembryos formed under all treatments of these experiments did not develop further [except in a few replicates (cotyledons) of the treatment 4mg/l 2,4-D] but either proliferated and fused together forming a callus mass or they turned from the white to brown colour without developing further (Fig. 13).

Table 4 shows the increase of the size of cotyledons under every treatment of the experiment in which BAP was used at concentrations 0, 0.1 and 1mg/l. As with adventitious bud and root formation, proembryo formation was induced very early and progressed while the cotyledon was expanding. Culture of cotyledons under continuous light or one week under dark and then continuous light did not show any difference in respect with morphogenetic responses. Tables 7 and 8 show the percentage of cotyledons which formed adventitious buds, roots and proembryos under different combinations of 2,4-D BAP and under different light conditions.

Table 7

Morphogenetic responses in cotyledon explants isolated from after-ripened embryos of apple var. Phiriki and cultured for 15 days on Pech *et al.* (1975) medium containing 2,4-D, BAP and combinations of them, under different conditions of light. Three values are given for each treatment. The first from the left is the percentage of explants which formed adventitious roots, the second buds and the third of explants which formed proembryos.

Light conditions	Concentration mg/l		2,4-D				
			0	0.2	2	4	8
One week dark and then continuous light	BAP	0	0 0 0	0 0 0	0 0 45	0 0 55	0 0 60
		0.2	0 0 0	10 0 0	0 0 55	0 0 70	0 0 75
		2	0 100 0	0 95 0	0 0 90	0 0 95	0 0 85
	BAP	0	0 0 0	5 0 0	0 0 45	0 0 45	0 0 70
		0.2	0 0 0	15 0 0	0 0 60	0 0 75	0 0 75
		2	0 100 0	0 100 0	0 0 85	0 0 95	0 0 95

Table 8

Morphogenetic responses in cotyledon explants isolated from after-ripened embryos of apple var. Phiriki and cultured for 15 days on Pech et al. (1975) medium containing 2,4-D, BAP and combinations of them, under continuous light. Three values are given for each treatment. The first from the left is the percentage of explants which formed adventitious roots, the second buds and the third of explants which formed proembryos.

Concentration mg/l		2,4-D				
		0	0.1	1	2	4
BAP	0	0 0 0	30 0 0	0 0 35	0 0 65	0 0 35
	0.2	0 0 0	5 0 0	0 0 90	0 0 75	0 0 95
	2	0 65 0	0 70 0	0 0 100	0 0 100	0 0 95

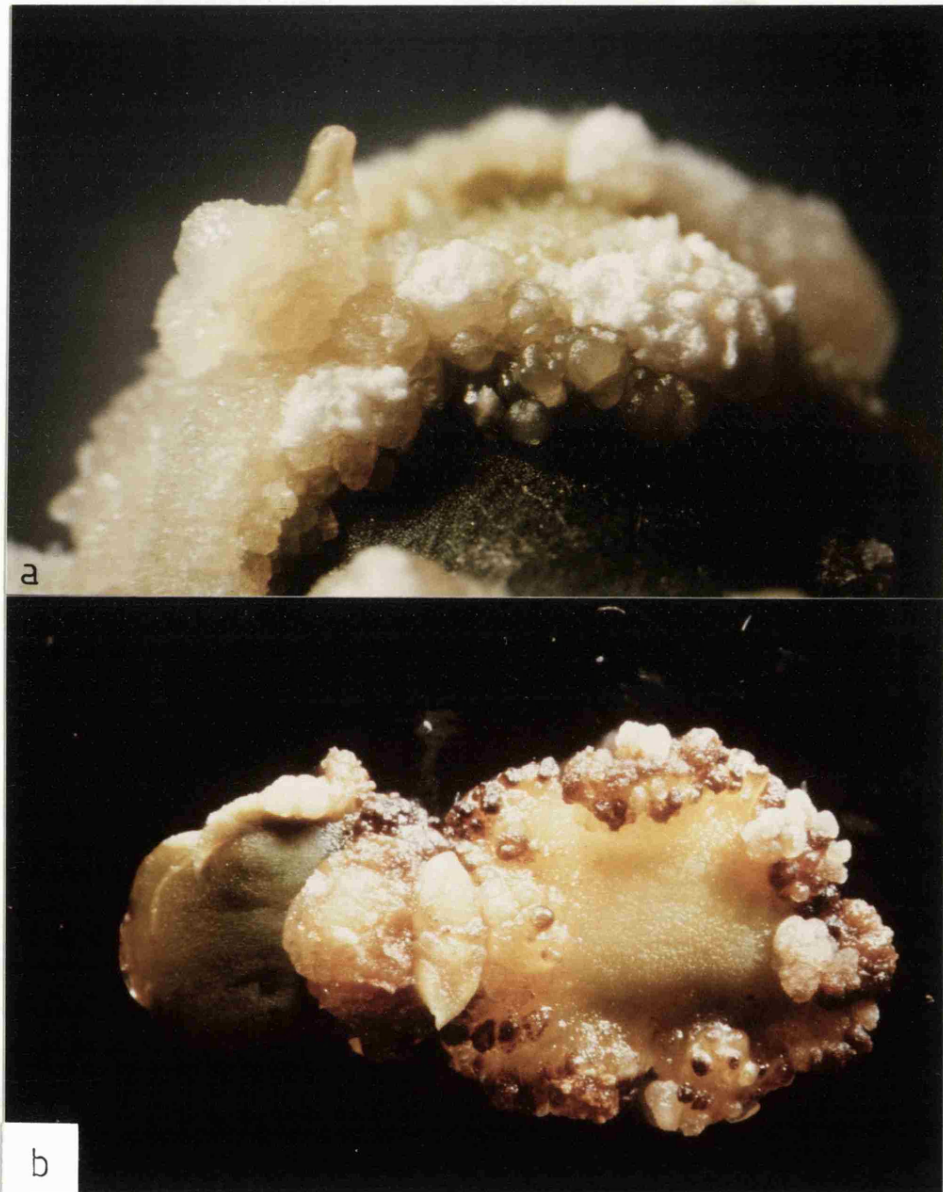


Fig. 13: a: An adventive embryo, proembryos and proembryos proliferated and fused together into a callus mass on a cotyledon explant isolated from apple embryo of the var. Phiriki and cultured on Pech et al. (1975) medium containing 4mg/l 2,4-D (x 15). b: Proembryos formed on cotyledons of embryo explants of the var. Phiriki cultured on Pech et al. (1975) medium containing 4mg/l 2,4-D either proliferated into callus or turned a brown colour without developing further (x 6).

b-2.2. Half cotyledons

The reasons for culturing half cotyledons have been explained in a-2.2 of this chapter. Cotyledons immediately after their isolation were cut in half and were cultured in combinations of 2,4-D/BAP at concentrations of 0, 0.2, 2, 4, 8 / 0, 0.2, 2mg/l respectively. Lower halves which included the petiole were cultured separately from the upper halves. As with intact cotyledons, half cotyledons started forming chlorophyll from the first day of their culture and on the third day they were dark green and expanded.

Lower halves responded morphogenetically like the intact cotyledons, the organs formed being localized at the petiole and seen after one week's culture. Adventitious buds continued their growth to shoots while proembryos either proliferated and fused together to a callus mass or turned into brown colour. Upper halves gave very poor morphogenetic responses in comparison to lower (Table 9 and Plate 14) and the organs mainly formed along the cut and close to the midrib (Fig. 14) could be seen after fifteen or twenty days culture. Adventitious buds did not develop further to shoots. Proembryos initiated on upper halves of cotyledons did not develop further as happened with proembryos formed on lower halves. Another difference between lower and upper halves was that the number of organs formed per half cotyledon was different. With upper halves there were one or two adventitious buds or proembryos while with lower halves and intact cotyledons as well there were many more and difficult to count.

Table 9

Morphogenetic responses in cotyledon explants isolated from after-ripened embryos of apple var. Phiriki and cultured for 15 days on Pech et al. (1975) medium containing 2,4-D, BAP and combinations of them under one week dark and then continuous light. Three values are given for each treatment. The first from the left is the percentage of explants which formed adventitious roots, the second buds and third of explants which formed proembryos.

Explant	Upper half of cotyledon					Lower half of cotyledon				
	2,4-D					2,4-D				
	0	0.2	2	4	8	0	0.2	2	4	8
BAP	0	0 0 0	0 0 0	0 0 30	0 0 55	0 0 0	0 0 0	0 0 0	0 0 10	0 0 5
	0.2	0 0 0	0 0 35	0 0 65	0 0 75	0 0 0	0 0 0	0 0 5	0 0 15	0 0 10
	2	0 80 0	0 75 0	0 0 100	0 0 95	0 0 100	0 0 0	0 0 10	0 0 15	0 0 15

Upper ?

lower ?

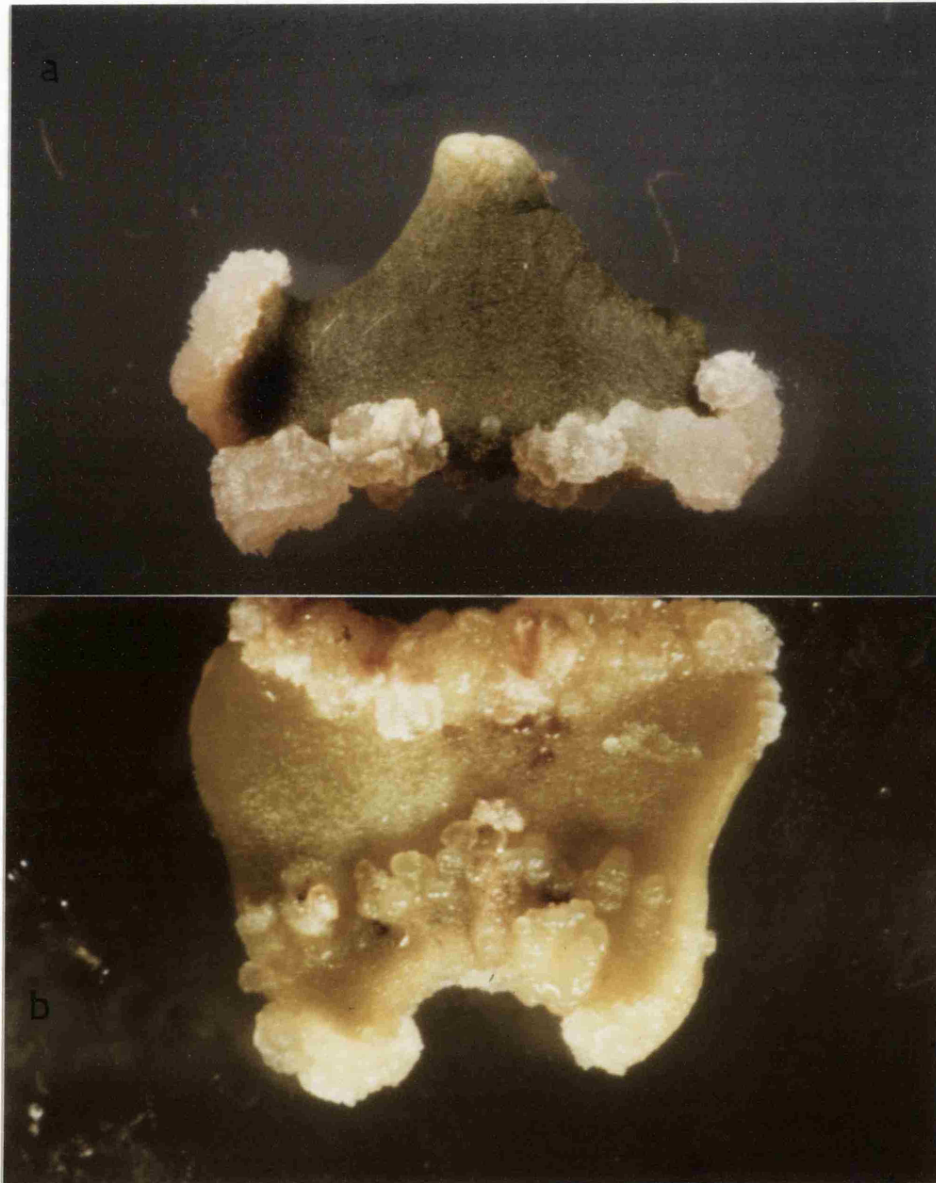


Fig. 14: Proembryo formation on upper (a) and lower (b) halves of cotyledon explants isolated from apple embryos of the var. Phiriki and cultured on Pech et al. (1975) medium containing 4mg/l 2,4-D. On the upper half there is only one proembryo formed in the area of the midrib (x 8).

b-3. Transfer from morphogenetic media to media permissive
of organ development

Most of the combinations of 2,4-D/BAP in the previous experiments induced proembryo formation. These proembryos did not develop further to embryos from which plants could be obtained.

As a first step for the further development of proembryos and adventitious buds formed on media containing combinations of 2,4-D/BAP, 16 replicates, embryos or cotyledons, from the previous experiments were transferred after 15 days on media containing combinations of 2,4-D/BAP at concentrations 0, 0.2 / 0, 0.1 and 0, 0.2 / 0, 0.2mg/l respectively. Four replicates remained on the first media. After two months no further development of proembryos to embryos and of buds to shoots was observed under any treatment of these experiments.

Because there was no difficulty in obtaining adventitious shoots by culturing cotyledons on media containing 2mg/l BAP in the following work emphasis was given to the development of proembryos to embryos from which possibly plants could be obtained. For this study the concentrations of 2, 4 and 8mg/l 2,4-D and the combinations of them with 0.2mg/l BAP were used. These treatments were selected because under them only proembryo formation occurred. There were 35 cotyledons (replicates) cultured in every treatment and five of them were transferred after 4, 8, 15 and 30 days on four media containing 2,4-D and BAP and combinations of them at concentrations of 0, 0.2 - 0, 0.2mg/l respectively. Although 25 cotyledons were enough because every treatment contained 5 replicates, there were 35 cotyledons per treatment because a certain number of them did not form any proembryos at all and was discarded. So all

cotyledons transferred and those which remained on the first treatments resulted in proembryos.

As it can be shown from the Table 10 embryos developed from proembryonic units mainly in the treatment 4mg/l 2,4-D and when cotyledons were transferred on the fourth day. The shorter the time the cotyledons were transferred the more embryos they developed. It seems that even four days culture on high concentration of auxin containing media is long enough to inhibit embryo development.

Embryoids do not germinate while they are attached to the cotyledon. All the embryos developed in this and previous experiments remained unpigmented and they did not continue their development to plants but proliferated and formed callus. The same happened to an embryo developed in one untransferred replicate of the treatment 4mg/l 2,4-D. Between the developed embryos and the maternal tissues there seemed to be no vascular connection as embryos were very easily detached by touching them. This was later confirmed by using micro-technique methods (g-1).

b-4. Embryo axis culture

The development of embryo axes cultured on media containing only BAP has been described previously.

Embryo axes were cultured on the same combinations of 2,4-D/BAP in which cotyledons were cultured. The first three-day embryo axes started developing towards plantlets having a distinct root, hypocotyl and shoot in the form of an apical bud with two or four leaves expanded. After one week's culture callus formation started

Table 10

Percentage of cotyledon explants isolated from after-ripened embryos of apple, var. Phiriki, and cultured on Pech et al. (1975) medium containing high 2,4-D concentration, which showed development of the proembryos to embryos after transfer to the same medium containing low 2,4-D concentration. The four values given in each treatment from left to the right show respectively the percentage of cotyledons in which embryo development occurred when transferred after 4, 8, 15 and 30 days to low auxin-containing media.

Concentration mg/l		2,4-D							
High auxin media	Low auxin media	2		4		8		0.2	
		0	0.2	0	0.2	0	0.2	0	0.2
0	0	0	0	0	0	0	0	1	0
	0.2	0	0	0	0	0	0	0	0
0.2	0	0	0	0	0	0	0	0	0
	0.2	0	0	0	0	0	0	0	0

from the different parts of the embryo axes, i.e., root hypocotyl and shoot meristem on treatments containing 2,4-D and combinations of it with BAP. Very often the whole embryo axis was developed to a callus. After fifteen days culture there was very little morphogenetic response in developed calluses. On the fifteenth day calluses were transferred on media of the same composition but containing lower concentrations of auxin and BAP. Controls were left on the first media.

There were totally 20 replicates per treatment. Four of them remained on the first media and the other 16 were transferred and distributed on the second media containing 0, 0.2-0, 0.1mg/l 2,4-D and BAP respectively and combinations of them.

The main characteristic of the calluses developed either originally or after transfer on media containing BAP was that they developed chlorophyll (Fig. 15a). Calluses developed on media containing 2,4-D and transferred on media containing 2,4-D or neither 2,4-D nor BAP were brown or white in colour,

Morphogenetic responses were very poor until the fifteenth day and observed in only two replicates of two treatments. The first was adventitious root formation in the treatment 0.1/0.1mg/l 2,4-D/BAP. An adventitious root arose from the base of a green leaf of the shoot meristem while adventitious buds were formed on the hypocotyl which together with the root were transformed into callus (Fig. 15b). The second was adventitious bud formation. Buds were formed on the upper part of the hypocotyl, which retained its normal shape while the remainder was transformed into callus. Two months after transfer root formation from callus occurred more often and on calluses developed originally on media containing 0.1 and 1mg/l

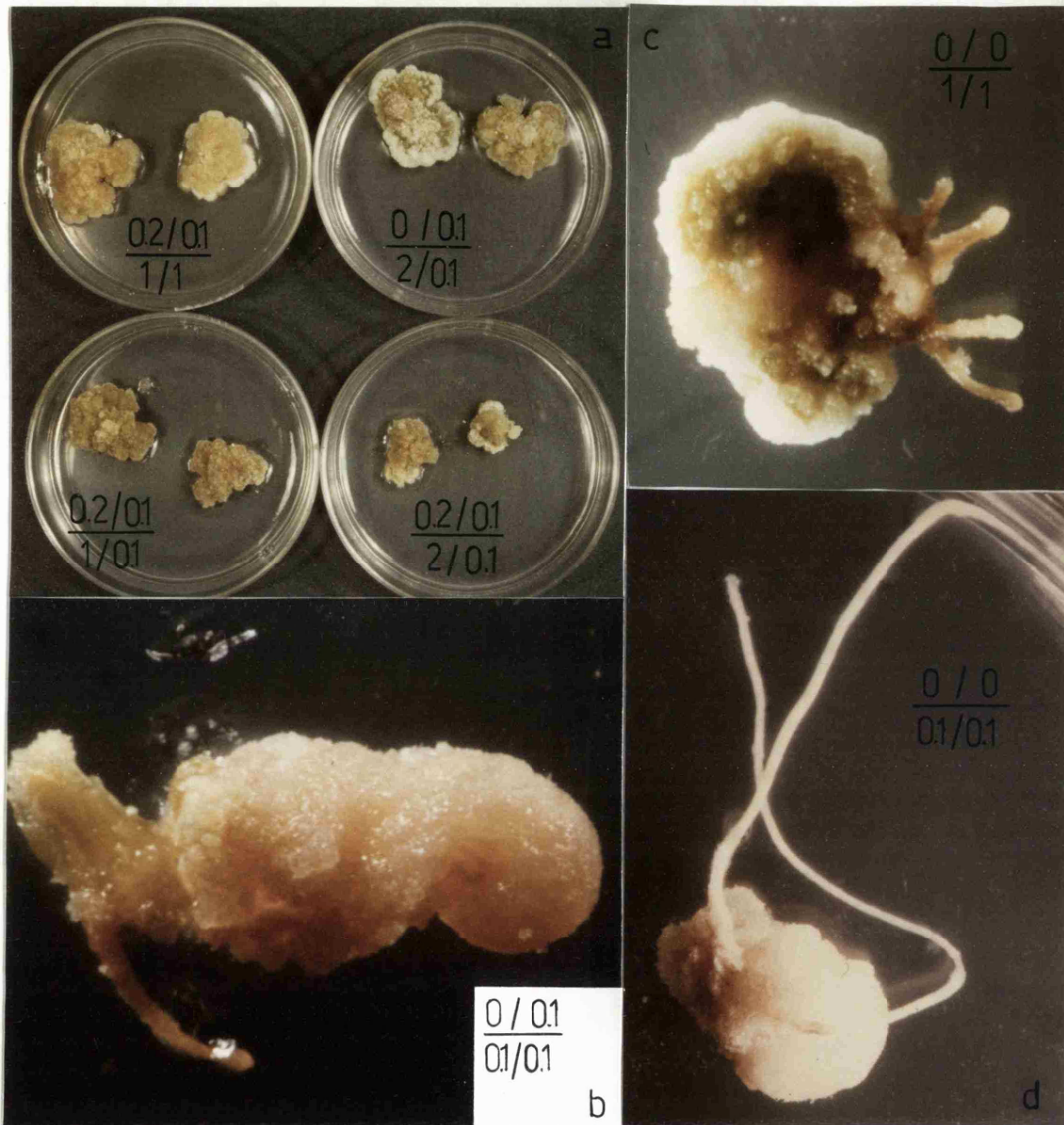


Fig. 15: Various responses of embryo axis explants isolated from apple embryos of the var. Phiriki when cultured on Pech et al. (1975) medium containing different combinations of 2,4-D/BAP. a: Chlorophyllous calluses. Each one has originated from a single embryo axis (x 0.8). b: Adventitious root formation from the base of the apical bud and adventitious bud formation on the upper part of the hypocotyl which together with the root has been grown to callus (x 9). c: Chlorophyll and adventitious root formation in callus originated from an embryo axis (x 20). d: Adventitious root formation (x 9). The combinations of 2,4-D/BAP on which embryo explants were originally cultured (denominator of the fraction) and those on which later were transferred (numerator of the fraction) are shown on each photo in mg/l.

2,4-D. Calluses developed originally on media containing 2 and 4mg/l 2,4-D did not show any morphogenesis. Among the calluses showing adventitious root formation (Fig. 15c-d) some were chlorophyllous. Adventitious root formation apart from calluses occurred also directly from leaves of the shoot which did not participate in callus formation. Calluses formed in the treatments 2/0.1, 1/0.1 and 2/1mg/l 2,4-D/BAP showed darker areas (Fig. 16a). Calluses having a nodular appearance were formed in the treatments 1/0.1, 4/0.1 and 4/1mg/l (Fig. 16b).

Embryo axes at the time of dissection weighed approx. 1mg. Three months after transfer there were calluses weighing more than 1g. This shows that embryo axes have a tremendous capacity to proliferate and form enormous amount of tissue in comparison to the original explant. In practice calluses did not grow any more two months after transfer. After this time only morphogenesis occurred.

More details about the colour and weight of calluses formed and morphogenesis before and after transfer are given in Table 11a-b.

c. INTERACTION OF 2,4-D-KINETIN

2,4-D and kinetin were combined at concentrations 0, 0.2, 2, 4, 8 and 0, 0.2, 2mg/l respectively. Only cotyledon culture was made and because morphogenetic responses were poorer in comparison to those under the interaction of 2,4-D-BAP no further investigation was made.

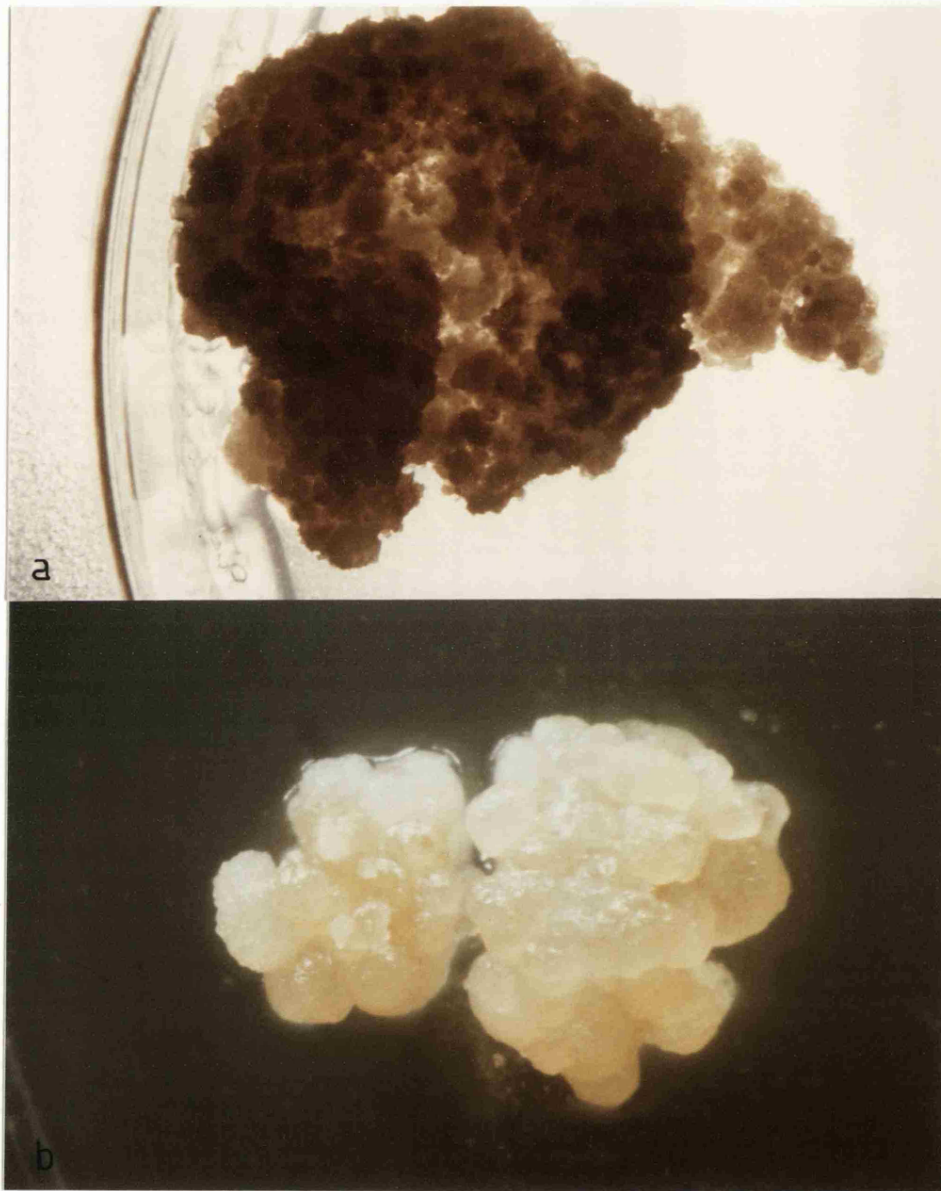


Fig. 16: Calluses originated from single embryo axes isolated from apple embryos of the var. Phiriki and cultured on Pech et al. (1975) medium supplemented with various combinations of 2,4-D/BAP. a: Callus grown on medium containing 2mg/l 2,4-D and 0.1mg/l BAP is showing darker areas. The photo has been taken by providing additional light underneath the callus (x 9). b: Callus grown on medium containing 4mg/l 2,4-D and 0.1mg/l BAP with nodular structure.

Table 11a

Growth and morphogenetic responses observed in embryo axis explants isolated from after-ripened embryos of apple var. Phiriki and cultured for 90 days on Pech et al. (1975) medium containing 2,4-D, BAP and combinations of them.

Concentration mg/l		2,4-D			
		0.1	1	2	4
BAP	0	Callus Brown Friable 0.270	Callus Brown Friable 0.130	Callus Brown Friable 0.08	Callus Brown Friable 0.07
	0.1	Callus Brown Friable 0.178 Adventitious* roots	Callus Brown Friable-Nodular 0.250	Callus White-green Friable 0.270	Callus White Friable-Nodular 0.200
	1	Callus Green Compact 0.120 Adventitious* buds	Callus Brown Friable 0.210	Callus White-green Compact 0.190	Callus White Friable-Nodular 0.200

* Adventitious roots formed at the base of developed meristematic leaves and adventitious buds on the upper part of the hypocotyl which did not participate in callus formation.

Table 11b

Growth and morphogenetic responses observed in embryo axis explants isolated from after-ripened embryos of apple var. Phitiki and cultured for 15 days on Pech et al. (1975) medium containing 2,4-D, BAP and combinations of them. Observations were recorded after 90 days from transfer of the explants to low 2,4-D and BAP concentration containing media.

Concentration mg/l	BAP	2,4-D							
		0.1		1		2		4	
		0	0.1	0	0.1	0	0.1	0	0.1
0		Development towards a little plantlet	Callus Green Compact 0.330	Callus (hypocotyl) * Brown Friable 0.100	Callus Brown Friable 0.150	Callus Brown Friable 0.09	Callus Brown-white Friable 0.010	Callus Brown Friable 0.08	Callus Brown Friable 0.09
0.2		Callus White-yellowish Compact 0.460	Callus Green Compact 0.392	Callus Brown Friable 0.270 Adventitious roots	Callus Green Compact 0.090	Callus Green Compact 0.172 Adventitious roots	Callus Brown Friable 0.226	Callus (shoot meristem) Green Compact 0.222	Callus Green-white Compact 0.142
0		Callus Green Compact 0.426 Adventitious roots	Callus Brown Friable 0.541	Callus Brown Friable 0.270	Callus Green Compact 0.200	Callus Brown Friable 0.140	Callus Brown Friable 0.170	Callus Green or Brown Friable 0.263	Callus Brown Friable 0.319
0.2		Callus Green Compact 0.724 Adventitious roots	Callus Green Compact 0.641	Callus Green Compact 0.530 Adventitious roots	Callus Green Compact 0.470	Callus Green Compact 0.500	Callus Green Compact 0.650	Callus Green Compact 0.415	Callus Green Compact 0.380
0		Callus (root) * Green Compact 0.184 Adventitious roots	Callus (hypocotyl root) * Green Compact 0.278	Callus Green Compact 0.101 Adventitious roots	Callus Green Compact 0.270	Callus White-green Compact 0.281	Callus White Friable 1.060	Callus Green Compact 0.153	Callus Green Compact 0.693
0.2		Callus (hypocotyl) * Green Compact 0.345	Callus (root) * Brown Friable 0.481	Callus Green Compact 0.375	Callus Green Compact 0.220	Callus Green Compact 0.190	Callus White-greenish Compact 0.672	Callus White Compact 0.837	Callus Green Compact 0.930

* Part of the embryo axis from which callus originated

c-1. Cotyledon culture

Cotyledons cultured on media containing 2,4-D/kinetin gave similar but poorer morphogenetic responses in comparison to those cultured on media containing 2,4-D/BAP. Under 0.2mg/l kinetin cotyledons gave no morphogenetic response. Adventitious buds were formed with 2mg/l kinetin. These buds developed very little, having two or four leaves expanded. Shoots long enough to be transferred on other media for rooting were not obtained. Adventitious roots were occasionally formed under 0.2mg/l 2,4-D. Structures very similar to proembryos formed on cotyledons cultured on media containing 2, 4, 8mg/l 2,4-D and combinations of them with 0.2mg/l kinetin. Apart from the proembryo formation under the same treatments white callus was formed along the edge of petiole. This callus proliferated and in some cases covered the proembryos formed on the petiole. When 2, 4 and 8mg/l 2,4-D were combined with 2mg/l kinetin proembryos were formed together with adventitious buds. Neither the proembryos nor the adventitious buds developed further. Adventitious buds were very tiny. They could be observed under the stereoscope as green structures and could be differentiated from proembryos which were white and bigger. Proembryos could be observed visually after 15 days. Callus was also formed under these treatments. The higher the concentration of 2,4-D the smaller the callus was.

Cotyledons cultured under continuous light or under dark for a week and then continuous light gave similar morphogenetic responses and the development of the organs formed was not affected by the different light treatments.

As with previous experiments adventitious organ formation was localized at the petiole of the cotyledons in these experiments as well.

In comparison to BAP, kinetin either alone or on combination with 2,4-D gave poorer morphogenetic responses. As it can be shown from Table 12 the percentage of cotyledons on which proembryos were formed was lower in comparison to that of BAP, as was the percentage of cotyledons on which adventitious buds were formed (Tables 6 and 16). Under BAP treatments adventitious buds developed further to shoots but under kinetin the adventitious buds developed very little and practically no shoots were obtained. The time adventitious buds and proembryos appeared was also different. With BAP treatments this time was one week while with kinetin it was 15 days.

d. INTERACTION OF NAA-BAP

From the previous experiments it is very clear that IAA induced root formation while 2,4-D induced proembryo formation. Two auxins had a different morphogenetic effect on the same tissues. In these experiments the effect of NAA either alone or in combination with BAP at concentrations 0, 0.2, 2, 4, 8 and 0, 0.2, 2mg/l respectively on morphogenesis of cultured cotyledons and embryo axes is studied. The experiments were carried out under continuous light and under one week dark and then continuous light.

d-1. Cotyledon culture

Chlorophyll formation and expansion together with the development of a distinct petiole were the characteristics of the cotyledon culture for the first three days. After 15 days there were

Table 12

Morphogenesis in cotyledon explants isolated from after-ripened embryos of apple, var. Phiriki and cultured for 20 days on Pech et al. (1975) medium containing 2,4-D and kinetin and combinations of them under different conditions of light. From the three values given in each treatment the first from the left shows the percentage of explants which formed adventitious roots, the second buds and the third of explants which formed proembryos.

Light conditions	Concentration mg/l		2,4-D				
			0	0.2	2	4	8
Continuous light	kinetin	0	0 0 0	0 0 0	0 0 30	0 0 40	0 0 45
		0.2	0 0 0	0 0 5	0 0 25	0 0 30	0 0 25
		2	0 15 0	0 20 0	0 0 45	0 0 55	0 0 55
One week dark and then continuous light	kinetin	0	0 0 0	5 0 0	0 0 45	0 0 45	0 0 55
		0.2	0 0 0	0 0 5	0 0 25	0 0 35	0 0 30
		2	0 20 0	0 15 0	0 0 35	0 0 40	0 0 45

morphogenetic responses in every treatment except the control and the treatment of 0.2mg/l BAP.

NAA alone induced adventitious root formation. Roots formed under NAA treatments were thicker and shorter in comparison to those formed under IAA treatments. In one cotyledon in the treatment 0.2mg/l NAA adventitious bud formation was observed and in another cotyledon in the treatment 8mg/l NAA simultaneous bud and root formation occurred. Formation of adventitious buds under only NAA was unexpected.

When NAA was combined with 0.2mg/l BAP mainly adventitious buds were formed. As it can be shown from the Table 13 the higher the concentration of NAA combined with 0.2mg/l BAP the higher the percentage of cotyledons having adventitious buds was. As well as adventitious buds, formation of adventitious roots and simultaneous formation of adventitious roots and buds occurred.

The same morphogenetic responses were observed when NAA was combined with 2mg/l BAP. Again adventitious buds were mainly formed while adventitious root formation occurred in the experiment set under dark for a week and then under continuous light. Simultaneous bud and root formation was observed as well at low percentages (Fig. 17). In contrast with IAA and 2,4-D which in combination with BAP inhibited the development of adventitious buds to shoots, shoot development from adventitious buds took place under every combination of NAA with BAP.

Between the combinations of NAA with 0.2 and 2mg/l BAP the difference is that adventitious bud formation with 2mg/l BAP occurred at high percentages as with the BAP alone. Although with

Table 13

Morphogenesis in embryonic explants isolated from after-ripened embryos of apple, var. Phiriki and cultured for 15 days on Pech et al. (1975) medium containing NAA, BAP and combinations of them. From the three values given in each treatment the first from the left shows the percentage of explants which formed adventitious roots, the second buds and the third roots and buds.

Explant		Cotyledon						Embryo axis					
Light Conditions	Concentration mg/l	NAA						NAA					
		0	0.2	2	4	8		0	0.2	2	4	8	
Continuous light	0	0	0	0	5	0	0	0	0	0	0	0	0
	0.2	0	0	0	10	0	0	0	0	10	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0
One week dark and then continuous light	0	0	0	0	5	0	0	0	0	0	0	0	0
	0.2	0	0	0	5	0	0	0	0	0	0	0	0
	2	0	0	0	5	0	0	0	0	0	0	0	0
Continuous light	0	0	0	0	5	0	0	0	0	0	0	0	0
	0.2	0	0	0	5	0	0	0	0	0	0	0	0
	2	0	0	0	5	0	0	0	0	0	0	0	0
One week dark and then continuous light	0	0	0	0	5	0	0	0	0	0	0	0	0
	0.2	0	0	0	5	0	0	0	0	0	0	0	0
	2	0	0	0	5	0	0	0	0	0	0	0	0

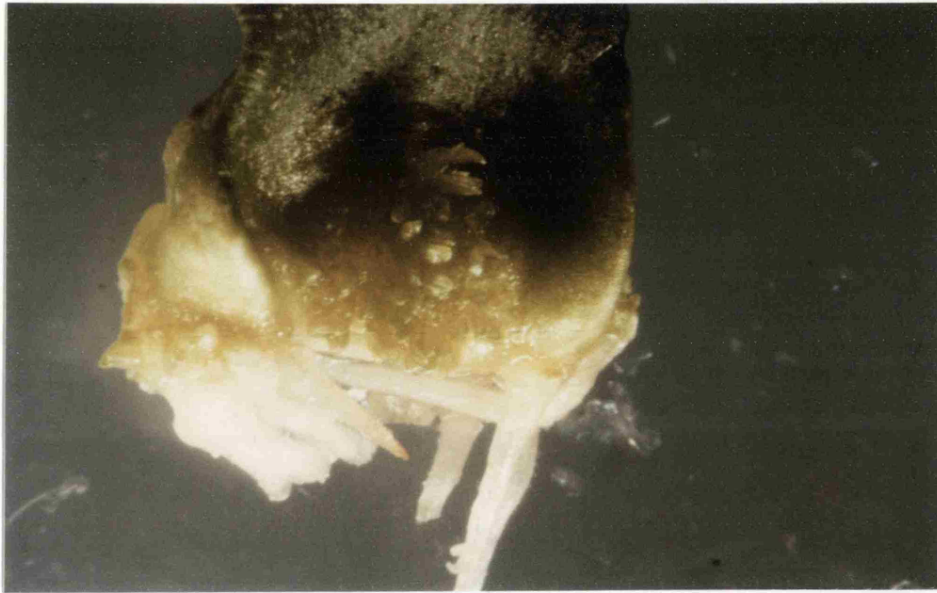


Fig. 17: Formation of adventitious buds and roots on cotyledon explants isolated from apple embryos of the var. Phiriki and cultured on Pech et al. (1975) medium containing 2mg/l NAA and 2mg/l BAP.

0.2mg/l BAP alone there was no morphogenetic response, there was when the same concentration of BAP was combined with NAA which indicates that NAA shows a synergistic-like effect with the BAP on the adventitious bud formation. This is confirmed first by the fact that adventitious buds formed under every combination of NAA/BAP continued their development to shoots and second by the fact that spontaneous bud formation occurred in cotyledons cultured under dark on media containing only NAA.

Apart from the localization of morphogenetic responses on the petiole of the cotyledons shown in this experiment as well another characteristic of the NAA-BAP treatment was that no callus was formed. Adventitious shoots arose directly (Fig. 18) from the green petiole and had strong vascular connections with the maternal tissue.

Table 14 shows the increase of the size of cotyledons after one month's culture.

In these experiments because all the adventitious buds continued their development to shoots there was no need to transfer cotyledons to other media allowing further development of shoots. Between different light treatments there were not any differences in respect to morphogenesis except that culture under one week dark and then under continuous light slightly favoured adventitious root and bud formation.



Fig. 18: Adventitious shoot formation on cotyledon explants isolated from apple embryos of the var. Phiriki and cultured on Pech et al. (1975) medium containing combinations of NAA/BAP. Note the complete lack of callus and the occasional rooting (x 2.5).

d-2. Embryo axis culture

The development of embryo axes and their morphogenetic responses when cultured on media containing 0, 0.2 and 2mg/l BAP has been previously described. Under 0.2 and 2mg/l NAA embryo axes developed towards plants having a shoot consisting of 2-4 leaves expanded, a hypocotyl and a root. In a small number of embryo axes between the hypocotyl and the root a small callus was formed and adventitious roots were arising from this callus. With 4 and 8mg/l NAA the shoot meristem developed to a shoot 0.5cm long, the hypocotyl grew into a callus mass from which adventitious roots were arising and the root either developed very little or remained undeveloped. The highest percentage of adventitious root formation was noticed with 4mg/l NAA (Table 13).

When the same concentrations of NAA were combined with 0.2mg/l BAP adventitious bud, root and formation of both occurred on the hypocotyl directly from the green tissue of the hypocotyl without any interfering callus. The main morphogenetic response was adventitious bud formation. Adventitious root formation and formation of both roots and buds happened at lower percentages. Small callus was formed in very few embryo axes between the hypocotyl and the root but it could not be related with any organ formation. The morphogenetic responses were mainly observed when NAA was combined with 2mg/l BAP. However adventitious ^{bud} formation occurred at higher percentages when NAA was combined with 2mg/l than with 0.2mg/l BAP. As is shown in Fig. 19 adventitious bud formation mainly occurred on the upper part of the hypocotyls and around the PCA. Buds under every treatment continued their development to shoots (Fig. 20).

Table 14.

Relevant increase of the size of cotyledon explants isolated from after-ripened embryos of apple var. Phiriki and cultured for 30 days on Pech et al. (1975) medium supplemented with NAA, BAP and combinations of them.

Light conditions	Concentration mg/l		NAA			
			0.2	2	4	8
Continuous light	BAP	0	5.02	3.64	4.50	5.17
		0.2	7.38	8.06	6.27	7.18
		2	9.43	8.08	8.04	- *
One week dark and then continuous light	BAP	0	4.24	3.66	3.00	5.06
		0.2	8.38	6.85	6.04	5.78
		2	7.46	7.50	9.91	6.83

* Contaminated



c

b

a

Fig. 19: Growth and morphogenetic responses of embryo axes explants isolated from apple embryos of the var. Phiriki and cultured on Pech et al. (1975) medium containing NAA and combinations of it with BAP. From the four lines the first from the top shows embryo axes cultured on medium containing 0.2mg/l, the second 2mg/l, the third 4mg/l and the fourth 8mg/l NAA. a: Shows the embryo axes cultured on medium containing 0mg/l. b: 0.2mg/l and c: 2mg/l BAP (x 2).

NAA

0.2

2

4

8



Fig. 20: Adventitious shoot formation on embryo axes explants isolated from apple embryos var. Phiriki and cultured on Pech et al. (1975) medium containing 2mg/l NAA and 2mg/l BAP (x 1.5).

Between the different light treatments there were almost no differences.

- e. Embryo, cotyledon and embryo axis culture on combinations of growth regulators selected from the previous experiments

All the previous experiments were carried out using seed of the cultivar Phiriki. These experiments included the culture of embryos, cotyledons and embryo axes explants isolated from after-ripened and dormant seed of the cultivars. Phiriki and Golden Delicious. The control in these experiments was intact cotyledons isolated from after-ripened seed of the cv. Phiriki. Apart from intact cotyledons, half cotyledons, lower and upper halves, and intact cotyledons with their upper surface in contact with the medium were cultured to see which part of cotyledon was responsive to morphogenesis and if position of the cotyledon had any effect on morphogenesis.

The growth regulators selected and the combinations of them were: 2mg/l BAP, 2mg/l IAA + 2mg/l BAP, 2mg/l NAA + 2mg/l BAP and 4mg/l NAA + 2mg/l BAP. The first and the last two were selected because they constitute a possible approach for plant regeneration. The second was selected to see if by using different explants or explants at different physiological status, adventitious buds could develop further to shoots in case simultaneous root and shoot formation was occurring.

It was thought that culture of embryonic explants on selected treatments which repeatedly and uniformly responded to morphogenesis and plant regeneration with similar tissues could be a good assessment of their morphogenetic capacity and potential for plant

regeneration before any complete experiment was set.

All the following experiments were carried out under dark for a week and then continuous light. There were twenty replicates per treatment.

e-1. Cotyledon culture

The morphogenetic results obtained under the different treatments and the percentage of cotyledons responded to them are shown in Table 15. Intact cotyledons and lower halves of cotyledons gave similar results in comparison to those obtained in previous experiments on the same combinations of growth regulators, as did cotyledons cultured with their upper surface in contact with the medium. Thus the position of the cotyledon in culture did not seem to have any effect on the expression of morphogenesis.

Upper halves of cotyledons cultured on the four selected media gave very poor morphogenetic responses confirming that petiole is the cotyledon tissue showing high morphogenetic capacity. Any adventitious buds formed on upper halves of cotyledons did not develop further to shoots.

The reactions of cotyledons isolated from dormant embryos were interesting, particularly that one in which the cotyledonary (axillary) bud sprouted forming a shoot up to 4cm long while at the base of this shoot a thick root, similar in appearance to the primary root of an embryo, was formed. A little later on the lower surface of the cotyledons and very close to the root adventitious buds were formed (Fig. 21). Under 2mg/l BAP cotyledons responded morphogenetically



Fig. 21: Cotyledon explants isolated from dormant apple embryos var. Phiriki and cultured on Pech et al. (1975) medium containing 2mg/l BAP. a: The cotyledonary bud has sprouted to a shoot at the base of which a thick adventitious root has been formed (x 2). b: As in (a) plus the adventitious bud formation (x 5). c: Adventitious root formation just below the shoot originated from the cotyledonary bud and just below the adventitious buds (x 2).

mainly in the previous way. Other morphogenetic responses were 1) formation of adventitious buds only; 2) formation of a long shoot from the axillary bud only and 3) formation of a thick root similar to the primary root of an embryo just below the adventitious bud (Fig. 21). When 2mg/l BAP was used in combination with 2mg/l IAA some of the cotyledons formed simultaneously adventitious roots and buds, some only adventitious buds and a smaller percentage only adventitious roots. When NAA was used in combination with 2mg/l BAP mainly adventitious bud formation occurred. Adventitious roots were formed together with adventitious shoots but at lower percentages. Under all treatments and regardless of any organ formation the axillary bud of the cotyledon sprouted and formed a shoot up to 6cm long. Adventitious buds formed continued their development to shoots under all previous treatments except that in which IAA was combined with BAP.

The main characteristics of NAA treatment was the complete lack of callus. From the data of the Table 13 it is shown that the percentage of cotyledons forming adventitious buds under every treatment was higher when BAP was combined with NAA. This is in agreement with previous experiments. With cotyledons isolated from dormant embryos the percentage of cotyledons forming adventitious shoots was higher in the presence of IAA and NAA while the percentage of cotyledons showing formation of both, shoots and roots, was decreased. This is opposite from what could be expected because it is known that auxins cause root formation and BAP as cytokinin has an inhibitory effect on root formation.

e-2. Embryo and embryo axis culture

Embryos and embryo axes regardless of physiological conditions (i.e. dormancy) and variety responded morphogenetically on the same way under the same treatments. With 2mg/l BAP mainly adventitious buds were formed. When the same concentration of BAP was combined with 2mg/l IAA adventitious buds, roots and mainly both were formed. NAA in combination with BAP resulted mainly in formation of buds but also formation of roots either alone or together with buds occurred at lower percentages (Table 15). With embryo axes organ formation was observed always on the hypocotyl and usually on its upper part and around the P.C.A. With embryos it was observed mainly on the hypocotyl and usually on the lower surface of the petiole of the cotyledon.

Embryos and embryo axes isolated from dormant seed in contrast with cotyledons, when cultured on media containing 2mg/l BAP formed adventitious roots rather spontaneously. Shoot malformation with secondary adventitious shoot formation was observed in one dormant embryo cultured under 2mg/l BAP (Fig. 22).

f. Regeneration of plants through adventitious shoot formation

Adventitious shoots were isolated for rooting two months after cotyledon and three months after embryo axis cultures were set. At this time shoots longer than 1cm were mature, neither soft nor lignified having well differentiated xylem elements. These conditions are known to be favourable for rooting. Another reason for isolating shoots at this time was also that shoots did not grow any further after two months culture for cotyledons and three months culture for

Table 15

Morphogenetic responses of various types of cotyledon explants isolated from apple embryos var. Phiriki and cultured for 15 days on Pech et al. (1975) medium supplemented with different growth regulators and combinations of them. From the three values given in each treatment the first from the left shows the percentage of explants which formed adventitious roots, the second buds and the third buds and roots.

Cultivar	Phiriki					Golden				
	1*	2	3	4	5	1	2	3	4	5
Treatment										
IAA/BAP 0/2mg/l	0 95 0	0 75 0	0 5** 0	0 100 0	0 0 70 0	0 70 0	0 65 0	0 0 0	0 90 0	0 0 80
IAA/BAP 2/2mg/l	20 25 55	5 20 40	0 15 0	10 45 45	15 15 55	0 40 50	10 30 55	0 5 0	15 35 50	20 35 40
NAA/BAP 2/2mg/l	0 75 15	0 70 30	0 0 0	0 80 15	0 95 5	0 50 10	0 80 15	0 0 0	0 90 10	0 85 15
NAA/BAP 4/2mg/l	0 70 30	0 90 10	0 0 0	0 60 40	0 90 10	0 75 25	0 70 30	0 0 0	0 65 25	0 80 20

- * 1: Cotyledons)
 2: Lower halves of cotyledons)
 3: Upper halves of cotyledons)
 4: Cotyledons cultured with their upper)
 surface in contact with the medium)
 5: Cotyledons isolated from dormant embryos)
 ** Foliar structures
- Isolated from after-ripened embryos



Fig. 22: Shoot malformation and secondary adventitious shoot formation observed a: in a dormant embryo explant of the var. Phiriki and b: in an adventitious shoot produced from a cotyledon explant isolated from an after-ripened embryo of the same variety. Both explants were cultured on Pech et al. (1975) medium supplemented with 2mg/l BAP (x 2, x 4).

embryo axes. This might have been either because of the depletion and dehydration of the nutrient medium or because of the genetic potential of the cultured tissues.

At the time of isolation shoots had the following characteristics. The majority of them had green leaves with small laminae and long internodes (Fig. 5). A small number had leaves unusually wide with a wide petiole and wide internodes from which small adventitious shoots were arising (Fig. 22). All these characteristics were attributed to the effect of BAP. Shoot malformation together with secondary adventitious shoot formation occurred mainly on media containing only 2mg/l BAP.

The concentration of 2mg/l IAA was selected for rooting of adventitious shoots because of the following reasons. First 2mg/l IAA gave the highest rooting percentage with cultured cotyledons (Table 2). Second the base of adventitious shoots where rooting was expected to occur and the petiole of the cotyledon which is very responsive to rooting under IAA treatments were of the same physiological status. Third in relation with the second it was clear from the previous experiments that rooting could take place under IAA treatments regardless of the presence of BAP in the medium. The average number of shoots obtained per cotyledon and embryo axis under selected treatments is shown in Table 17. The highest number of shoots obtained per explant was achieved when 2mg/l NAA was combined with 2mg/l BAP.

Shoots transferred to media containing 2mg/l IAA formed adventitious roots. Table 18 shows the percentage of shoots which

Table 16

Morphogenetic responses of embryo and embryo axis explants of apple var. Phiriki cultured for 30 dayson Pech et al. (1975) medium supplemented with different growth regulators and combinations of them. From the three values given in each treatment the first from the left shows the percentage of explants which formed adventitious roots, the second buds and the third buds and roots.

Explant	Embryo						Embryo axis					
	Phiriki			Golden			Phiriki			Golden		
Treatment	1	2	2	1	2	2	1	2	1	1	2	2
IAA/BAP 0/2 mg/l	0 60 0	0 55 0		0 75 0	0 70 0		0 65 0	0 50 10	0 80 0	0 70 5		
IAA/BAP 2/2 mg/l	10 10 80	5 10 45		15 20 65	5 5 55		5 5 65	5 15 60	10 15 70	10 10 55		
NAA/BAP 2/2mg/l	0 70 0	0 60 5		5 85 0	0 50 5		5 95 0	0 90 10	0 85 15	0 95 5		
NAA/BAP 4/2mg/l	10 20 70	0 65 15		15 10 70	0 55 25		15 85 0	0 80 20	0 80 20	0 75 25		

1: After-ripened or isolated from after-ripened embryos

2: Dormant or isolated from dormant embryos

Table 17

Number of adventitious shoots obtained per cotyledon and embryo axis explants isolated from after-ripened embryos of apple var. Phiriki and estimation of the number of adventitious shoots obtained per embryo when cotyledons and embryo axes cultured for two and three months respectively on Pech et al. (1975) medium supplemented with various growth regulators and combinations of them.

Treatment	Number of shoots developed per										Number of shoots obtained per embryo			
	Cotyledon					Embryo axis								
	2cm>	>1-2cm>	>1cm	Total	2cm>	>1-2cm>	>1cm	Total	2cm>	>1-2cm>	>1cm	Total		
	2cm>	>1-2cm>	>1cm	Total	2cm>	>1-2cm>	>1cm	Total	2cm>	>1-2cm>	>1cm	Total		
2mg/1 BAP	7.10	5.24	5.98	18.32	1.45	3.75	0.86	4.61	15.65	14.23	12.82	42.70		
2mg/1 IAA + 2mg1 BAP	0	0	0	0	0	0	0	0	0	0	0	0		
2mg/1 NAA + 2mg/1 BAP	8.77	6.01	5.35	20.13	7.82	4.92	4.18	16.92	25.36	16.94	14.88	57.18		
4mg/1 NAA + 2mg/1 BAP	7.30	4.82	5.80	17.92	4.10	3.90	4.02	12.02	18.70	13.54	15.62	47.86		

Table 18

Percentage of adventitious shoots originated in cotyledon and embryo axis explants of apple var. Phiriki, which rooted after transfer on Pech et al. (1975) medium containing 2mg/l IAA, and percentage of those which rooted and continued their development to a plantlet.

Shoot length at time of excision	Percentage of rooted shoots	Number of roots per shoot	Percentage of shoots developed to plantlets
2cm >	45	5.8	45
>1-2cm >	35	4.1	30
>1cm	20	2.3	10

formed roots, the number of roots formed per shoot and the percentage of rooted shoots which developed to plantlets. As it can be shown under the 2mg/l IAA treatment the highest rooting percentage 40% was obtained with shoots longer than 2cm and the lower 20% was with shoots shorter than 1cm. Shoots longer than 2cm responded better and from the point that a) higher number of roots were formed per shoot and b) that all of them which formed roots developed to little plantlets (Fig. 23). Among rooted shoots which were shorter than 1cm long some died after rooting and others showed inhibition of shoot development obviously because of the auxin concentration. Malformed shoots because of the BAP effect did not usually form roots.

Roots always appeared at the base of the shoots three weeks after transfer to media containing IAA.

Shoots treated as softwood cuttings died and disintegrated. This probably was because of the toxic effect of the alcohol contained in the IAA solution with which shoots were treated and the poor control of humidity in the plastic boxes covered with a polyethelene sheet.

No effort was made to transplant the little plantlets and transfer them to the soil.

g. Microtechnique

In previous work the existence of a cuticle around plant embryos was discovered. Cuticles are a product of secretion of epidermal cells. Although plant embryos are in a very early stage of cellular differentiation they are not devoid of an epidermis. Apart

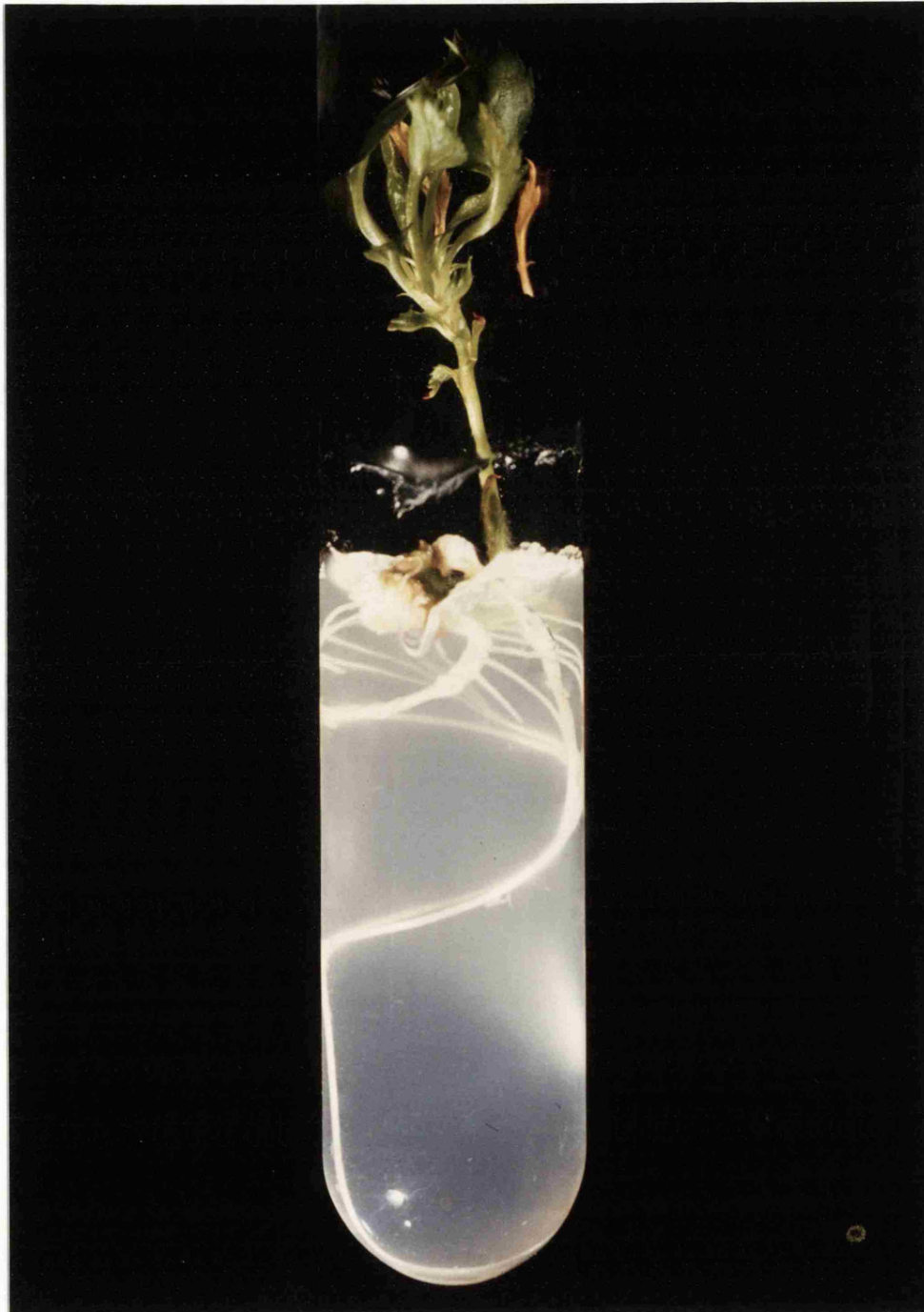


Fig. 23: Rooting of adventitious shoots on Pech et al. (1975) medium supplemented with 2mg/l IAA and production of plantlets. Adventitious shoots were produced on cotyledon explants isolated from apple embryos of the var. Phiriki and cultured on the same medium containing 2mg/l BAP.

from the cuticle which speaks for the existence of an epidermis this has also been demonstrated by microtome sectioning. Fig. 24 shows that embryonic cotyledons of apple cv Phiriki are surrounded by a mono-layer of cells which are smaller 5-10 μ m in size in comparison to others which vary in their size from 20-60 μ m. This layer of cells is described as epidermis. The same was observed in sections of embryo axes. The common characteristic of all the embryonic cells including the epidermis is the big internal organelles and the dense cytoplasm.

g-1. Origin of adventitious organs

Adventitious organs formed on the petiole of apple cotyledons originated from epidermal cells. Mitotic activity in all histological sections was confined to the epidermis of the petiole. Epidermal cells were activated, became more densely cytoplasmic and started dividing mitotically (Fig. 25). The pattern of bud and root formation was different from the pattern of embryo formation. As it can be shown from Fig. 26, in bud and root formation cell divisions started from epidermal cells and were extended to the tissue below the epidermis while at the same time the organ primordia were emerging as an outgrowth. In embryo formation though cell divisions started from the epidermis and they were not extended to the tissue below epidermis but embryos had the form of an outgrowth attached to the epidermis (Fig. 27)). These observations explain the fact that roots and buds were strongly connected with the maternal tissue while embryos had none or very little connection because they were detached just by touching them. No vascular connection between the embryooids and the maternal tissue could be detected in this work. In Fig. 26a-b

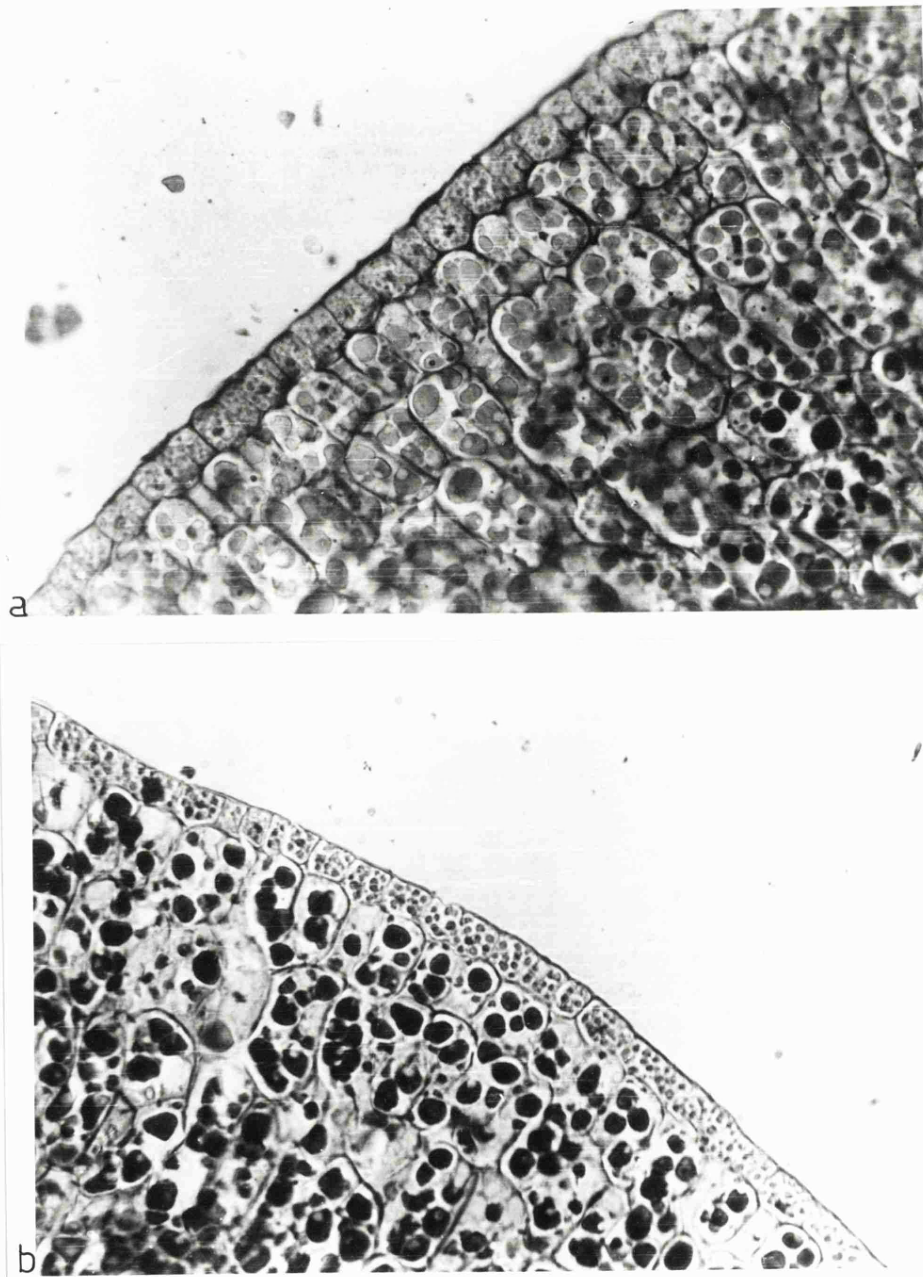


Fig. 24: Epidermis of a: cotyledon (x 700) and b: embryo axis (x 500) isolated from apple embryos of the var. Phiriki.

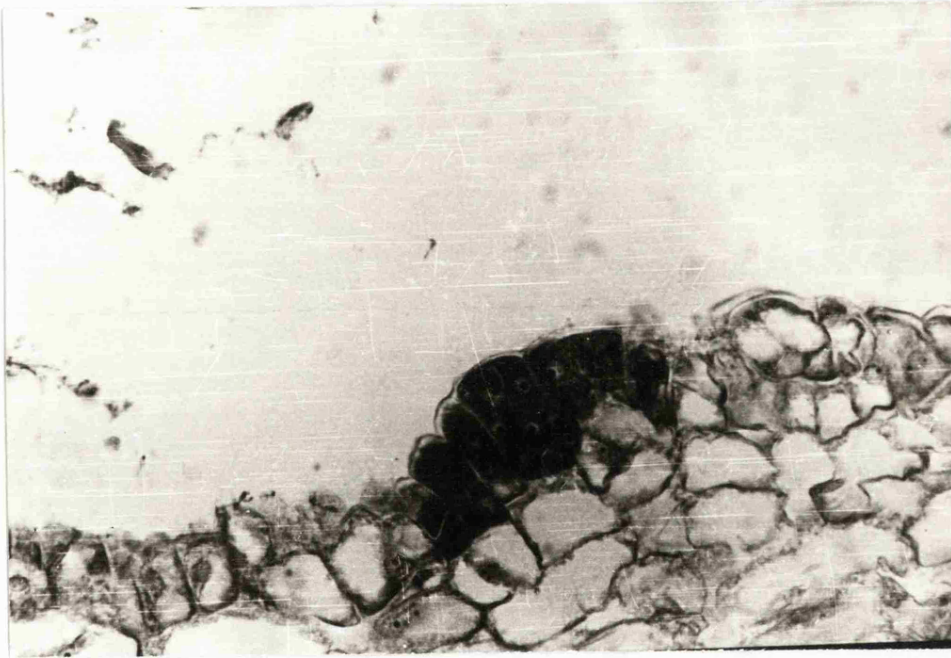


Fig. 25: Epidermal cells which became more densely cytoplasmic and started dividing mitotically (x 500). This is the way morphogenetic processes began in the petiole of cotyledon explants of apple var. Phiriki and Golden Delicious, when cultured on Pech et al. (1975) medium supplemented with various growth regulators.

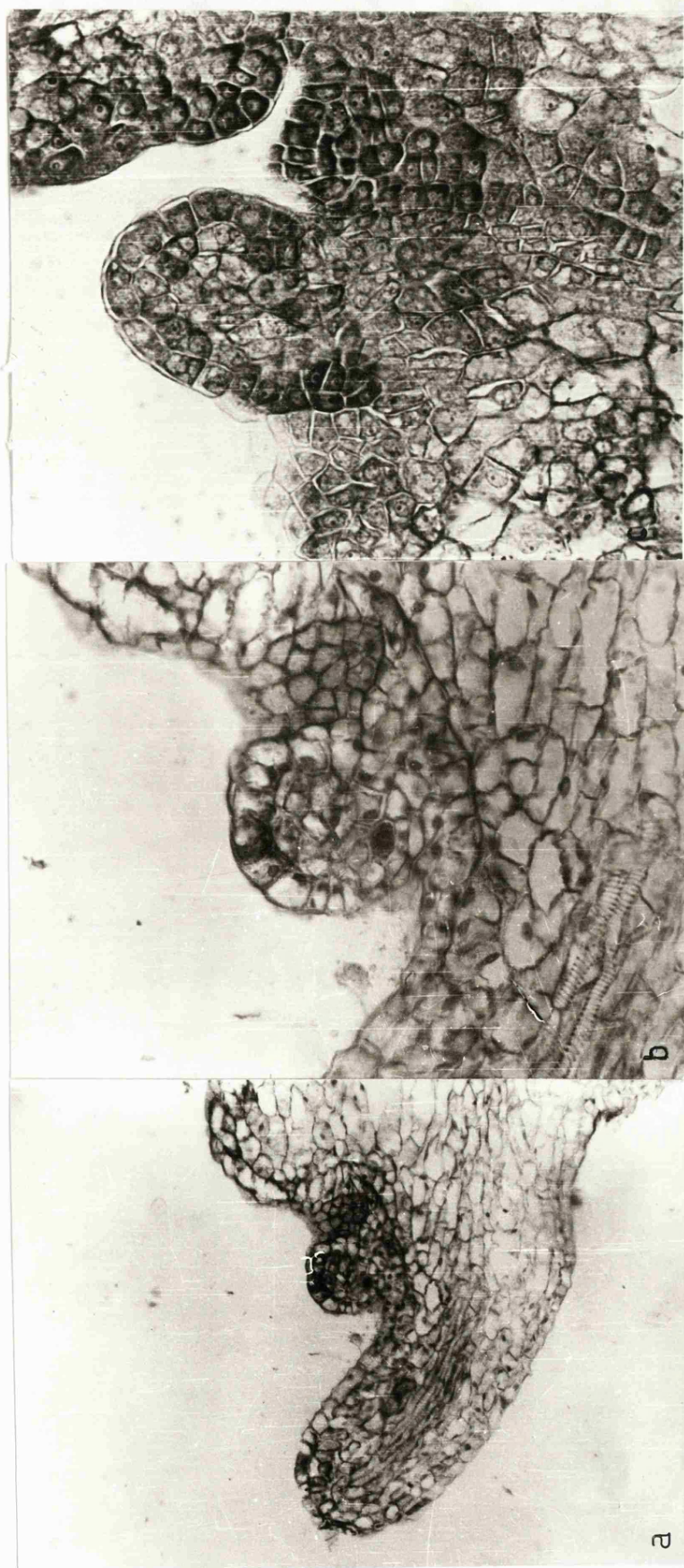


Fig. 26: Adventitious root and shoot formation on the petiole of cotyledon explants isolated from apple embryos of the var. Phiriki and cultured for six days on Pech et al. (1975) medium containing 2mg/l IAA for root or 2mg/l BAP for bud initiation. a: Emerging adventitious roots. b: Detail of a. In both differentiation of vascular tissue can be seen. c: Emerging adventitious buds. As with adventitious roots the participation of sub-epidermal tissue in the formation of adventitious buds is distinct (x 200, x 400, x 400).

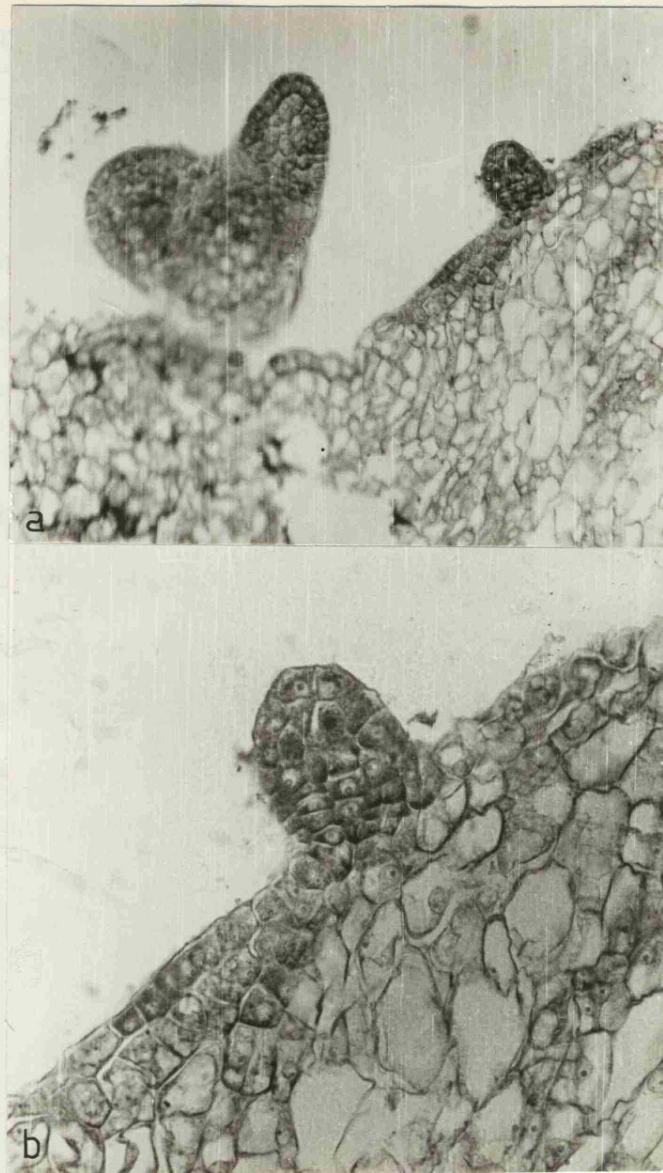


Fig. 27: Adventitious embryo formation on the petiole of a cotyledon explant isolated from apple embryos of the var. Phiriki and cultured for six days on Pech et al. (1975) medium supplemented with 4mg/l 2,4-D. a: An emerging embryo and a heart-stage embryoid. b: A detail of a showing the origin of the embryo from the epidermis without participation of the subepidermal tissue (x 200, x 400).

showing an emerging root from a petiole, differentiation of cells similar to those of vascular tissues can be seen.

From all the sections made it was concluded that organ formation was not synchronous. This was also confirmed by the fact that while adventitious shoots were already well differentiated new ones were arising.

g-2. Axillary buds of cotyledons

Fig. 28 shows the axillary bud of a cotyledon from the cv. Phiriki and cv. Golden Delicious. The difference in the shape of the buds is not because of the cultivar but it is rather because the section responds to a different position in the bud.



Fig. 28: Axillary buds of cotyledons of apple seedlings. a: of the var. Phiriki. b: of the var. Golden Delicious (x 300). The growth of buds was stimulated by decapitating the seedlings above the cotyledons. Samples were fixed a week after decapitation.

C. DISCUSSION

From the results obtained using IAA, BAP and combinations of them it is obvious that IAA induced adventitious root formation and BAP adventitious bud formation. When these two growth regulators were combined formation of both root and bud occurred. Buds however did not develop further to shoots.

Skoog and Miller (1957) working with tobacco callus cultures showed that two substances, an auxin and a cytokinin, were necessary for tissue growth and the pattern of organogenesis was determined by their relative concentrations in the nutrient medium. Initiation of root and shoot was basically regulated by interactions of these two regulators. A relatively high concentration of auxin favoured root formation and repressed shoot formation. In contrast, relatively high concentrations of cytokinin induced shoot formation and suppressed rooting. Equal concentrations of auxin and cytokinin resulted in callus formation. Murashige (1974) reported that the control of root and shoot initiation by auxin-cytokinin balances appears to be a general phenomenon among plants and it can be seen even in the mosses.

In this work it seems that root and shoot initiation was regulated by the exogenously supplied auxin and cytokinin respectively and that it was not determined by their relative concentrations. This is confirmed by the fact that when IAA and BAP were combined at equal concentrations (2mg/l) callus formation did not occur but root and shoot formation was observed simultaneously and independently.

In spite of this evidence the possibility that the control of root and bud initiation in embryonic tissue cultures of apple may be regulated by the auxin-cytokinin relative concentrations can still be supported. Polyakova (1977) found that IAA is present in seeds of apple and certain other fruit species, traced changes in its content in apple seeds during the process of stratification and found that germination of seeds after stratification was accompanied by a decrease in the content of this hormone. The presence of free IAA in seeds of apple was also found by Mousdale and Ward (1981). Barthe (1979) studied the endogenous levels of free and bound cytokinins in dormant and after-ripened embryos of the cv. Golden Delicious and showed that complex qualitative changes were also induced during the after-ripening treatment. Isaia and Bulard (1978) and Le Page-Degivry and Bulard (1979) showed the presence of gibberellins and abscisic acid respectively in embryos of apple cv. Golden Delicious. The presence of all these growth regulators in the embryos of apple indicates that the control of morphogenetic processes may not be dependent only on the exogenously supplied auxin and cytokinin and supports the idea that the control of morphogenesis may depend on the relative concentrations of endogenous and exogenously supplied regulators particularly auxins and cytokinins. Even if this is true the question which remains to be answered is how formation of both root and shoot is induced independently and simultaneously under different balances of auxin-cytokinin. The most probable explanation is that exogenously supplied IAA and BAP which very distinctively induced root and shoot formation respectively could allow morphogenetic processes to occur regardless of the presence of the other. In this work it was proved that adventitious root formation occurred and the roots developed normally in the presence of BAP

(2mg/l). IAA did not affect the initiation of adventitious buds but inhibited their development to shoots. These results are different in comparison to others obtained with embryonic tissues of other species. Cheng (1976) for instance found that morphogenetic responses in vitro of cotyledon explants of Tsuga heterophylla were strongly influenced by the interactions between exogenously added auxin and cytokinin.

Murashige (1974) developed the concepts of developmental stages and separated them into three steps for clonal multiplication. The first stage includes the isolation of the explant and its establishment in aseptic culture, the second the multiplication of the propagule and the third the establishment of rooted plants and their preparation for transfer to soil. Each stage has specific requirements concerning the nutrient medium and the environmental conditions. Different nutrient media may be necessary for each stage or usually one medium can be employed throughout the first and the second stages.

Apple seeds are in a deep dormancy and stratification at chilling temperatures 4-7°C is necessary for up to three months. In this work apple seeds were the explants. They were easily isolated but before the embryonic tissues were isolated from them and established in cultures, they had to after-ripen. This postmaturation of the seed and consequently of the embryo was carried out on an agar layer checking at the same time for successful sterilization. Working with embryonic tissues isolated from dormant seed could be another shorter way. It would certainly be more reasonable though for a research worker to start with postmatured embryos, investi-

gate the factors controlling morphogenesis and then apply them to embryonic tissues isolated from dormant embryos.

There was no difficulty in establishing the explants (first stage) in aseptic culture. The Pech et al (1975) medium supplemented with 2mg/l BAP was successfully employed throughout the first and the second stage for the production of adventitious shoots with cotyledon and embryo axes explants. When the explants were intact embryos the same medium containing 2mg/l BAP induced bud primordia but failed to support their further growth and development. It was obvious that there was an explant-hormone interaction. Different explants can respond to ingredients of a medium in various ways. This has been very well demonstrated by Sharp et al (1973). In their work, seed, fruit, plagiotropic and orthotropic shoot, leaf and anther explants of coffee responded in various growth and differentiation patterns when cultured on the same medium containing 2,4-D. Not only can different organs give different results but sometimes portions of a single explant may behave differently. Powell (1970) for example reported that the basal portion of an apple stem is more dormant in comparison to the upper portions and therefore may give different results in vitro.

When the Pech et al. (1975) medium was supplemented with 2mg/l BAP plus various concentrations of IAA, bud primordia were formed but their development to shoots was inhibited. IAA either supplemented at low concentrations (0.1mg/l) or at higher 2mg/l had the same repressing effect on bud development. The explanation for this can be that endogenous IAA in cotyledons of apple embryo may be at an already high or optimum concentration for the initiation of buds and any addition of exogenous IAA results in suppression of bud

development. This hypothesis explains also the fact that adventitious shoots were formed on cotyledon and embryo axis explants cultured on medium supplemented with BAP only. The shoot formation might be because of the relative concentrations of exogenously supplied BAP plus the endogenous cytokinins interacting with the endogenous IAA. Mehra-Palta et al. (1978) working with cotyledon explants of Pinus taeda found that high auxin concentrations were inhibitory to bud induction, but low concentrations acted synergistically with cytokinin so that every cotyledon formed buds. BAP alone is also known to have induced formation of adventitious buds on cotyledon and other explants of Biota orientalis and other species of the Cupressaceae (Thomas et al., 1977) and on excised cotyledons of Pinus taeda embryos (Mehra-Palta et al., 1978).

While IAA had an inhibitory effect on bud development, BAP unexpectedly did not have any inhibitory effect on rooting. When the medium was supplemented with 2mg/l IAA almost every cotyledon formed roots regardless of the presence of BAP. Cytokinins are known to prevent root initiation and inhibit root growth. Skirvin (1980) reported that rooting is frequently inhibited by the presence of kinin. In this work root growth of cultured embryos was inhibited by the presence of high concentrations (1 or 2mg/l) BAP, while the same concentrations of BAP caused sprouting of the lateral buds including the axillary buds of cotyledons. In this case there was no apical dominance, lateral shoots were formed and the total shoot systems had the form of a rosette. On the contrary low concentrations favoured better root growth in comparison to controls. BAP also, at low concentrations and in combination with 2mg/l IAA, favoured secondary root formation from adventitious roots formed in cotyledon explants.

Since cytokinins inhibit rather than favour root formation perhaps the BAP induced the formation of meristematic centres along the main roots which later (under the effect of IAA) developed to secondary roots.

Because BAP induced bud initiation in the presence of IAA but the growth of buds to shoots was inhibited by IAA in separate experiments, the explants were transferred from the first medium which was the bud formation inducing medium on other media containing low concentration of IAA and BAP and on control media containing no IAA and BAP. The percentage of explants showing bud formation increased after transfer (Table 5). This was because the stimulation for bud initiation was given on the first medium and buds appeared on the second medium. Neither the buds formed before transfer before transfer nor the buds which appeared after transfer developed further to shoots. This showed that neither lowering the auxin concentration nor the bud formation after transfer on the low auxin and control media resulted in further growth of buds to shoots. Mehra-Palta et al. (1978) induced the growth of adventitious buds formed on cotyledon explants of Pinus taeda working in the following way. 1) Dissected buds or bud clusters usually with a small part of the cotyledonary tissue were planted on bud growth media. 2) The buds initiated on a cytokinin/auxin combination were transferred to a medium lacking these growth factors. 3) A half strength basal medium was used which stimulated bud growth. 4) 1% charcoal was incorporated into the half strength medium. Charcoal stimulated further the growth of buds. 5) High light intensity (9,000 lux) was applied which proved to be an important factor for the good growth of the shoots. From this procedure only the second step was used in this work with apple. Although

the same procedure could be applied to see if it could stimulate the growth of buds formed in apple cotyledons under IAA/BAP combinations: there was emphasis given because adventitious shoots could be formed under 2mg/l BAP either alone or in combination with NAA. Cotyledon explants when IAA and BAP were incorporated into the medium at 2mg/l each, formed well developed roots and buds. If these buds could develop further to shoots then possible rooted plants could be obtained during the second stage by separating a shoot together with a root. It would therefore be interesting to see if buds or bud clusters dissected together with a root could be developed to a rooted plantlet by following the way Mehra-Palta et al. (1975) worked. In addition the use of auxin antagonists could possibly overcome the suppressive effects of an excessive supply of endogenous auxin. Although these procedures would result in a smaller number of plantlets produced per embryo than rooting adventitious shoots it might be of economic importance because it minimizes the third stage to the preparation of the propagule for transfer to soil.

Cotyledon explants in culture formed chlorophyll and grew bigger from the first day of their culture. It was not the purpose of this study to find out if the growth of cotyledons was because of cell division or cell expansion or both. However I think that the growth was because of cell expansion and that cell division occurred only in the area of the cotyledon which was determined to form the petiole. This is the same area in which morphogenesis occurred. In cotyledon explants cut at their base, i.e. having the area which was determined to form the petiole removed, morphogenesis occurred at lower percentages and at a lower intensity (number of organs formed

per explant). The development also of adventitious buds was inferior because they did not grow to shoots and the roots were thin and short. The same were observed with upper halves of cotyledon explants. Cheng (1977) working with Douglas fir cotyledons divided the entire length of the cotyledon into three equal parts described as basal-middle- and distal regions according to its position from near the stem axis towards the proximal end. She found that basal regions were more responsive to in vitro stimuli for differentiation than those obtained from the other two regions. This is in agreement with the results of this work where the basal part of the cotyledon, i.e. the petiole gave better morphogenetic responses than cotyledons cut at their base and which in turn responded better than the upper halves of cotyledons. In this work cells from the cotyledon were already differentiated and responded poorly to morphogenesis while those determined to form the petiole were not differentiated, they were capable of active division and contained a high morphogenetic capacity.

The observation that morphogenesis occurred while the cotyledon was growing showed that morphogenetic processes started before or while cells of the base of the cotyledon were dividing to form the petiole. Cotyledons at the time of excision had no distinct petiole which was formed at a later stage of the cotyledon development. Figure 29 shows the pattern excised cotyledons grew in culture. It is obvious that formation of new tissue and the differentiation of the petiole occurred at the base of the excised cotyledon while the remaining part grew or expanded retaining its original shape. It was expected that actively dividing cells could respond better to morphogenesis than partly or fully differentiated ones.

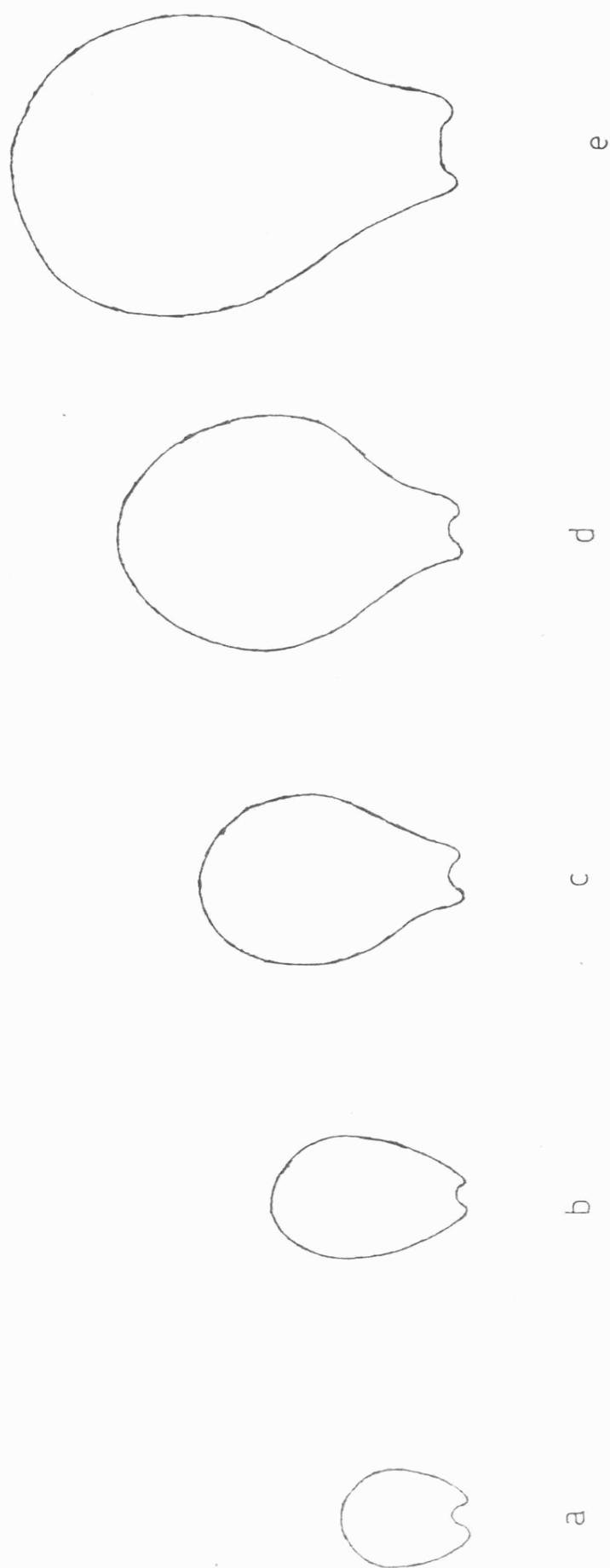


Figure 29: The pattern coteledon explants isolated from apple embryos of the varieties Phiriki and Golden delicious grew in agar cultures. a: cotyledon at the time of excision. e: after fifteen days of culture on Pech et al. (1975) medium. b, c, d: intermediate stages. Scale ≈ 3 .

Cells from petiole tissue responded differently to morphogenesis under different growth regulators. Under IAA they responded in rooting and under BAP to shoot formation. What is difficult to explain is how cells of the same small tissue region (petiole) can differentiate, the stimuli given by two different growth regulators and respond to one of them, and how neighbouring cells can respond to root or shoot formation when IAA and BAP were incorporated in the medium. Are there any physiological-biochemical differences among neighbouring cells of the petiole due to which cells can respond only to one of the two stimulations given and what is the mechanism of rejecting the other? Certainly the answer to these questions is related to the way auxins and cytokinins work at the molecular level which is yet unknown.

Under the IAA BAP treatments organs originated either directly from the maternal tissues of the explant or from callus. IAA alone or in combination with low concentrations of BAP induced callus formation. With cotyledon explants callus was formed along the edge of the petiole or cut of the cotyledon and its formation was independent of any organ initiation. With embryo and embryo axis explants IAA induced callus formation 1) from the area between the hypocotyl and the root when applied at low concentrations (0.1-0.2mg/l) 2) from the hypocotyl and the root when applied at high concentrations (2mg/l). From these calluses only adventitious roots arose while adventitious buds originated directly from maternal tissues without any intervening callus.

Exogenous auxin stimulation can cause the initiation of callus. From embryos or embryo axes explants cultured on media containing IAA only the root and the hypocotyl responded with callus formation. With 2mg/l IAA the whole part below the PCA was transformed into callus while the part above the PCA including the cotyledons retained its usual shape and continued its normal development. Porlingis (1973) reported that roots, buds and shoots have a different sensitivity to the auxins and that the optimum concentration of auxin for their growth is different for each one. The optimum concentration of auxin required for shoot growth is higher than that required for bud growth. The optimum concentration for root growth is lower in comparison to those for shoot and bud growth. This report explains why only the root and the hypocotyl responded in callus formation under the concentrations of IAA used in this work. Perhaps concentrations of IAA higher than 2mg/l would affect the whole embryo or embryo axis and convert it into a callus mass.

2,4-D had a different morphogenetic effect in comparison to IAA. Spontaneous root formation was observed in the treatments 0.2/0 and 0.2/0.2mg/l 2,4-D/BAP respectively. Proembryo formation occurred under higher concentrations than 1mg/l 2,4-D, either alone or in combination with BAP. 4mg/l 2,4-D only was optimum concentration for the initiation of proembryos capable of developing further after transfer. From the results obtained by transferring explants at different times (Table 10) it is obvious that the shorter the time of culture on the first media the more the percentage of cotyledonary embryos obtained. Transfer even four days after inoculation seems to be too long a time. Shorter times i.e. one day or a few hours culture on 4mg/l 2,4-D containing media and the incorporation of charcoal in

the low auxin containing media should be tested for obtaining higher percentages of fully developed embryos. Charcoal perhaps adsorbs excessive growth regulators which may otherwise affect the normal growth of embryos. After a method for producing high percentages of fully developed embryos has been established transfer of embryos to new media should be tested for their further development to plants.

All the proembryos and embryos formed in cotyledon and embryo explants of this work remained unpigmented throughout their culture. Hu and Sussex (1971) noticed that young embryoids initiated on the cotyledons of Ilex aquifolium cultured embryos were colourless or green depending on the state of pigmentation of the cotyledon cells from which they were initiated. Some which were initially colourless remained unpigmented so long as they were attached to the cotyledon, but others were synthesizing chlorophyll as they were enlarging.

2,4-D is the most widely used auxin for the induction of embryogenesis. Mott (1980) described the usual procedure for the induction of embryogenesis on 2,4-D/kinetin-containing media and Kohlenbach (1978) in his review reported that although the general scheme can vary among species, most cases involve two steps. One with high auxin concentration for induction and a second with auxin removal for embryo development. Reports of somatic embryogenesis in tree species are rare in comparison to those of herbaceous plants. Mott (1980) reported that with the exception of Citrus and Coffea species, there is a lot of work to be done in making somatic embryogenesis a reliable avenue for vegetative propagation and he quoted a table of thirteen trees and woody species in which somatic embryogenesis occurred in vitro. Stamp and Henshaw (1982) reported somatic

embryogenesis with Cassava (Manihot esculenza Crantz) cotyledon explants induced on Murashige and Skoog (1962) medium supplemented with 4mg/l 2,4-D only. Transfer after 24 days on the same medium deprived of auxin resulted in embryo development. Plantlets were produced on the same medium from somatic embryos.

Proembryos were formed in the petiole of the cotyledons but their formation was not so strictly localized as bud formation. With cotyledon explants when BAP was used at 2mg/l in combination with 2,4-D adventitious bud primordia which could not be visually observed were formed together with proembryos. How bud primordia and proembryos could be formed independently in the presence of BAP and 2,4-D is a question similar to that commented earlier on bud and root formation in the presence of BAP and IAA. With respect to bud formation 2,4-D suppressed bud development more than IAA. This was expected because 2,4-D is a stronger synthetic auxin in comparison to IAA, an auxin naturally existing in plants.

Embryo axes cultured on 2,4-D-containing media either alone or in combination with BAP responded mainly in callus formation. The whole embryo axis or part of it proliferated and formed a callus mass. Morphogenetic responses, mainly root formation, were very poor and observed either directly from maternal tissue or through callus.

Embryo axes were very likely to respond by callus formation because of two reasons. First they are very small in size. Okazawa et al. (1967) found that smaller explants are more likely to form callus while the larger explants maintain greater morphogenetic potential. Second, although any part of a plant can be induced to produce a callus, juvenile tissues are most likely to succeed. What

is important to be emphasized here is the tremendous ability of embryo axes to proliferate. At the time of inoculation embryo axes weighed approximately 1mg and after two months culture some of them were heavier than 1g (Table 11b). Gamborg and Shyluk (1981) reported that after 3-4 weeks culture the callus should be about five times the size of the explant, and Cheng (1975) working with Pseudotsuga menziesii small explants (2mm in length) reported that the fresh weight of tissue had increased at least 50-fold over that of the original inoculum. In this work the explant weight could be increased by a factor of 1000 and more. These results are different from those obtained by Mehra and Sachdeva (1979). In their work excised embryos without cotyledons, i.e. embryo axes never formed callus on variously supplemented media.

Callus growth was better and had the tendency to form chlorophyll in the presence of BAP. Coloured calluses were not formed in the presence of only 2,4-D. Aitchison et al. (1977) reported that many factors other than light are known to affect the chlorophyll content of tissue cultures, including iron availability, auxins and kinins and sugars, especially sucrose. From the works of Newmann (1962), Bergmann and Bälz (1966), Israel and Steward (1966), Newmann et al. (1969) and Neumann and Raafat (1973) it is clearly shown that under normal tissue culture conditions, callus cultures are known to develop chloroplasts in the light, carry out photosynthesis and evolve oxygen. However, the presence of sucrose in the culture medium inhibits both chlorophyll synthesis and photosynthetic carbon fixation in tissue cultures (Neumann and Raafat, 1973). Formation of chlorophyllous calluses from apple tissues has also been reported by Mu et al. (1977) and Mehra and Sachdeva (1979). Other species from which chlorophyllous

calluses have been obtained are tobacco (Laetsch and Stetler, 1965), Atropa (Davey, Fowler and Street, 1971) and carrot (Hanson and Edelman, 1972).

The cytokinins usually used in morphogenetic experiments are BAP, 2iP, kinetin and Zeatin. Their effectiveness varies from one tissue to another. Mehra-Palta et al. (1978) working with Pinus taeda cotyledon explants found that Zeatin and BAP were more effective in inducing bud formation than 2iP and kinetin. Thomas et al. (1977) reported that of the three cytokinins tested (BAP, kinetin and Zeatin) only BAP was effective in inducing adventitious bud formation irrespective of the basal medium. Zee and Hui (1977) found that kinetin in combination with NAA appeared to be a bit more effective in inducing root and shoot formation from excised cotyledons of Brassica alboglabra than the BAP-NAA combination. Cheng (1977) reported that 2iP was least effective in inducing adventitious bud formation in Douglas fir cotyledon cultures and that although kinetin was slightly less active than BAP for the initiation of bud primordia it resulted in significantly more rapid formation of needle primordia. Based on these results Cheng suggested that combined use of BAP and kinetin could be advantageous. Webb and Street (1977) found that BAP either alone or in combination with 2iP caused maximal expression of bud induction in P. sitchensis. In this work kinetin was found to be less effective than BAP first in inducing adventitious bud formation and second in inducing proembryo formation in combination with 2,4-D.

NAA showed a synergistic effect with BAP on the induction of bud and shoot formation. There are many reports about NAA in respect with the induction of adventitious buds giving various and

opposite results. Zee and Hui (1977) reported that if NAA (2mg/l) was used alone, it could induce plantlet formation from excised cotyledon segments of chinese kale with well developed root system. Shoot formation though tends to proceed at a slower rate in comparison with the NAA (4mg/l) + kinetin (0.5mg/l) combination which was found to be optimum. In this work adventitious bud formation occurred in the presence of NAA only (Table 13) but it was spontaneous. Brown and Sommer (1977) reported that with Monterey pine (Pinus radiata D. Don) a combination of 5mg/l BAP and 0-0.05mg/l of NAA was effective in inducing buds on the cotyledons or hypocotyls of mature embryos. Different results were obtained by Thomas et al. (1977). They found that NAA in combination with 6-BAP reduced the percentage of buds formed. Gunay and Rao (1978) found that combination of BA and IAA were very effective in inducing bud formation in cotyledon explants of Red pepper but when NAA was substituted for IAA bud development was completely suppressed and explants produced scanty whitish callus. Cheng (1977) working with Douglas fir cotyledon explants found that the extent to expression of morphogenetic potential was greatly influenced by the concentration of exogenous auxin incorporated into the culture medium in combination with BAP. At nanomolar level, NAA stimulated maximum bud production while at macromolar level it inhibited all bud production and the cultures grew rapidly as friable calluses.

The three auxins used in this work allowed different morphogenetic processes to occur. Explants responded also differently to these three auxins in respect to callus formation. The most and the least effective were 2,4-D and NAA respectively while IAA had an

intermediate effect. With embryo axes cultures 2,4-D induced mainly callus formation but IAA and NAA allowed morphogenesis to occur. Similar results were obtained by Gunay and Rao (1978) with cotyledon and hypocotyl explants of Capsicum. Under IAA and NAA explants formed callus and roots but under 2,4-D only callus was formed. In their work, though, NAA was more effective in inducing callus than IAA which caused mainly rooting. Lane (1982) also reported that NAA promotes callogenesis more strongly than IBA or IAA.

It is generally accepted that callus formation is antagonistic to root and shoot proliferation and for this reason most tissue culturalists strive to minimize callus. It is also well known that the use of callus cultures increases the probability of cellular variation (Skirvin, 1978) and therefore callus should be minimized to ensure clonal stability and maximize shoot and/or root proliferation. In this work shoots were initiated in cotyledon explants with exogenously-supplied BAP either alone or in combination with NAA without any callus formation. The increase of the size of the cotyledons was also different under each auxin combined with BAP. A maximum increase, approximately ten times fold was caused under NAA. IAA caused a four times fold increase and 2,4-D five times fold. With 2,4-D this increase was observed only under 0.1mg/l 2,4-D. Higher 2,4-D concentrations resulted in a smaller increase of the cotyledon. It seems that cotyledons have a capacity to increase in size not only when isolated directly from seed but also when isolated from seedlings. Bajaj and Nietsch (1975) reported that cotyledons excised from one-week old seedlings, when cultured on Murashige and Skoog (1962) medium, continued to increase in size and in some cases, they became ten times

larger than the original inoculum.

Formation of foliar structures was occasional and observed under IAA and 2,4-D treatments and usually in the lower surface of cotyledons. In comparison to embryoids foliar structures were green and they did not develop any further. Foliar structure formation has also been observed on cotyledon explants of Cassava (Stamp and Henshaw, 1982).

Studies on adventitious bud formation in culture have involved considerable variation in the physical cultural conditions adopted. In respect to light for instance embryos and cotyledon explants of P. menziesii have been successfully maintained under continuous light (Cheng, 1975) and under photoperiods of 16 (Winton and Verhagen, 1976), 18 and 12h (Cheng, 1977). Shorter photoperiod (12h) was more effective on the induction of bud production than longer photoperiod (18h) when cotyledons were cultured on 5nM NAA and 5 μ M kinetin. In this work, between the two different light conditions involved there were no significant differences on the promotion of adventitious root, shoot and embryo formation although one week culture under dark and then continuous light slightly increased the percentage of rooted explants and explants forming adventitious shoots.

Although rooting of adventitious shoots of apple still remains a limiting factor in the clonal propagation of some root-stock and scion cultivars there seemed to be no difficulty with rooting of adventitious shoots originated in embryonic tissues of cv. Phiriki and cv. Golden Delicious. Perhaps adventitious shoots formed in cotyledon explants of this work inherited juvenile char-

acters of the embryonic tissues they were produced from. Simple transfer to the same medium containing 2mg/l IAA resulted in 40% rooted shoots being originally longer than 2cm. Certainly this percentage could be significantly increased if other methods and techniques already established by other scientists were tested.

Anderson (1980) has summarised the main cultural factors which could be beneficial for root initiation. These factors are:

- 1) low salt concentrations in the medium. Lane (1978) for example had better results on rooting by transferring shoots to a medium containing half the normal concentration of salts.
- 2) Selection of the appropriate auxin and its concentration.
- 3) Addition of adsorbents such as activated charcoal.
- 4) Reduction of agar concentration to the lowest level but capable of supporting the plantlets.
- 5) Illumination with red light which according to Seibert (1973) stimulated root initiation. Abbot and Whiteley (1976) increased the percentage of rooted shoots from 30 to 80% by placing the IBA treated shoots in an inverted position on the medium.

Jones (1976, 1979), Jones et al. (1977, 1979) found that phloroglucinol had a beneficial effect on rooting of apples. These results concerning the effect of phloroglucinol could not be reproduced by Nemeth (1981) who found that the synthetic auxins 2-chloro-3-phenyl-propionitriles with some apple stock varieties were more or equally effective on adventitious root induction in comparison with IBA. Snir and Erez (1980) achieved 100% rooting with MM 104, MM 106 and MM 109 by scalpel incision (wounding) and incorporation of 0.25% charcoal in the rooting medium.

In this work none of these techniques has been employed to increase the percentage of rooting shoots because of limited time, but it has been clearly demonstrated that shoots of the cultivars Phiriki and Golden Delicious can easily form roots. The fact that shoots mainly shorter than 1cm died out after rooting was attributed first to the tender nature of their tissues and second to the auxin concentration which affected the growth of rooted shoots being shorter than 2cm. Dutcher and Powell (1972) found that high concentrations of IAA reduced shoot growth and Lane (1982) reported that excessive auxin concentrations should be avoided because they induce more root initials but they inhibit root extension and shoot growth. From the results obtained here it seems that the same concentration of IAA affected differently shoots at different stages of their development in two ways. First the percentage of rooted shoots was higher with longer shoots and second the shorter the shoot the more the inhibitory effect of auxin finally resulting in death of the smaller shoots.

Vegetative propagation of trees is beset with general problems associated with the perennial and woody habit of trees and the same can be said for vegetative propagation by way of tissue culture. Correlative inhibitions and seed and bud dormancy are fundamental to the tree habit and they may be an underlying cause for the general difficulty in getting good regeneration of plants in culture. Apple is a species which in comparison to other fruit species is characterized by a deep seed and bud dormancy. In this work embryonic tissues isolated directly from dormant seed responded equally well to morphogenesis in comparison to tissues isolated from after-ripened embryos. Furthermore with cotyledons isolated from dor-

mant seed morphogenetic responses were richer. Unexpectedly in the presence of 2mg/l BAP exogenously supplied, apart from the growth of the axillary bud and the adventitious bud formation, root formation occurred at the base of the shoot grown from the axillary bud. The impression was that cotyledons isolated from dormant seed in culture had the tendency to reproduce the missing embryo axis, i.e. to regenerate the whole embryo capable of further growth and development. It is difficult to explain how rooting occurred in the presence of only BAP exogenously supplied because there is very little research work concerning morphogenetic studies with tissues isolated from dormant trees. The simplest explanation could be that root formation occurred because of the endogenous regulators affected by the exogenously supplied BAP.

Adventitious shoots produced in the presence of BAP had small lamina, long internodes and some of them were abnormally swollen. Small lamina and long internodes are the main characteristics of etiolated shoots. Although the light intensity in this work was low (1000 lux) these morphological characteristics should be attributed to the BAP effect. Similar results in adventitious shoot morphology of apple were obtained by Jones (1967) and Pieniazek (1968) reported that elongation of the axis by BAP was unexpected because only auxins and gibberellins were considered to stimulate stem elongation. Pieniazek also reported that lateral and collateral shoots were abnormally swollen in the presence of 2mg/l BAP because of the marked development of the cortical parenchyma. Jones used a relatively high light intensity (5,300-5,500 lux) and Pieniazek used low light intensity (1,000 lux). The fact that adventitious shoots of apple showed

the same morphological characteristics under different light intensities indicates that the inducing factor is the BAP and not the light intensity.

It was shown in this work that adventitious organs formed at the petiole of apple cotyledon explants originated from epidermal cells. As mentioned earlier the existence of the embryo cuticle shows that embryo cells are progressed in differentiation. If epidermal cells are in a more advanced stage of differentiation in comparison to other embryonic cells, morphogenesis would be expected to occur in other embryonic cells except epidermal because differentiation and morphogenetic capacity are negatively correlated. If in spite of the supposed higher degree of differentiation morphogenesis occurs in epidermal cells, then another factor or factors could be responsible for the induction of morphogenesis from epidermal cells. Perhaps the stimuli given by endogenous and exogenous regulators are effective on epidermal cells only and embryo cuticle might play a certain role on it. However the fact that morphogenetic processes started from epidermal cells indicates that these cells are less differentiated in comparison with the other embryonic cells.

In this work it was found that adventitious buds originated from the epidermal as well as the sub-epidermal layers, while embryoids originated from the epidermal layer. In respect of adventitious buds similar results were obtained with Torenia fournieri hypocotyl and leaf explants in which adventitious buds originated from epidermal and sub-epidermal cells (Reinert et al., 1977). Similar results were also obtained in respect of adventitious embryo formation by Hu and Sussex (1971), who reported that for embryoids developed in cotyledon

explants of Ilex aquifolium there was evidence that they were not formed in a deep region of the tissue.

CHAPTER 5

CULTURE OF APPLE EMBRYO CELLS

A. INTRODUCTION

To complement the investigation on inducing organogenesis in apple embryonic tissues an attempt to induce organogenesis was made in cell cultures of apple embryo origin. Secondary liquid cultures and primary liquid and agar cultures were involved in this study. Four media were selected for studying the growth of cells and for supporting any morphogenesis in secondary liquid cultures. These media were 1) The Pech et al. (1975) medium which is a modification of the Murashige and Skoog (1962) medium. 2) The Gamborg et al. (1968) or B₅ medium. 3) The Shenk and Hildebrant (1972) and 4) The White's (1963) medium. The reason for selecting these media was that they have most often been used for plant regeneration (Evans et al., 1981). One more reason for using the White's (1963) medium was to follow the same protocol of work used by Ammirato (1974, 1977) for rearing embryos from apple embryo cells. In Ammirato's work somatic embryos were reared from cells of Caraway (*Carum carvi* L.) cultured in White's (1963) medium after transfer to Murashige and Skoog (1962) medium lacking the auxin. For agar cultures the same media were used except that instead of the White's medium, the Fujimura and Komamine (1975) medium was used. The reason for using this medium was to compare the growth behaviour of apple and carrot embryo cells when cultured in the same medium. As a disadvantage of the primary cultures of this work could be considered the heterogeneity of the cells which in every experiment were isolated from a relatively large number of embryos. According to Thomas and Davey (1975,a) homogeneous populations are subject to less variation than heterogeneous populations and biologically are the most meaningful for experimental

studies. In spite of the biological importance of homogeneous cell populations the use of heterogenous cell populations could be equally important if plant regeneration from them was feasible, (The carrot work described in Chapter 2 is a first step towards this direction) and if clones of single cell origin could be derived. This is further supported by the view that heterogeneity exists or appears in cell populations of a single explant origin (Thomas and Davey, 1975a). Furthermore what is a disadvantage under certain conditions might be an advantage under different conditions. For example heterogeneity or lack of genetic stability in plant regeneration through tissue cultures is a disadvantage for the nurseryman who has to reproduce true to type plants but it is certainly interesting and desirable for the plant breeder.

B. MATERIALS AND METHODS

a. Secondary cultures

a-1. Initiation of callus cultures of apple

Embryos from dormant and after-ripened apple seed of the varieties Phiriki and Golden Delicious were isolated aseptically and cultured on four media for callus formation. These media were 1) The Pech et al. (1975) medium containing a) 100mg/l casein hydrolysate, 1mg/l 2,4-D and 0.1mg/l BAP. b) 100mg/l casein hydrolysate, 2mg/l IAA and 0.1mg/l BAP. 2) The White's (1963) medium containing 1% coconut milk, 200mg/l casein hydrolysate, 2mg/l 2,4-D and 0.1mg/l BAP. 3) The Gamborg et al. (1968) or B₅ medium containing 1mg/l 2,4-D and 0.1mg/l kinetin and 4) The Shenk and Hildebrant (1972) medium containing 0.5mg/l 2,4-D and 0.1mg/l kinetin. The chemical composition of these media is given in Appendix 2.

For the preparation of the media inorganic components mixed with agar (Difco Bacto agar) were autoclaved at 121°C for 15 min, cooled at 30-40°C and mixed with the filter sterilized organic components. The final agar concentration was 0.8% and the pH (adjusted before sterilization) for the four media was respectively 5.8, 5.8, 5.5 and 5.9.

Except in the case of embryo explants hypocotyl explants of the two varieties were used for the induction of callus formation. Hypocotyls were isolated from seedlings which grew at 4°C after stratification of seed on bacteriological agar. They were cut to 1-1.5cm lengths and cultured on the same media. Explants were cultured in 9cm diameter Petri dishes containing 20ml of nutrient

medium. Cultures were set under dark or low light provided by a 40 watt bulb as previously described at $24 \pm 2^{\circ}\text{C}$. There were twenty replicates (embryo or hypocotyl explants) per treatment distributed in five Petri dishes.

a-2. Callus maintenance and subculture

Among the calluses developed from embryos, two for each medium and combination of growth regulators, one of the var. Phiriki and the other of the var. Golden Delicious, were selected for subculture. These calluses were maintained on medium of the same composition and subcultured regularly every 20 days. There were totally eight callus cultures from two varieties maintained on four media. The two cultures developed on Pech et al. (1975) medium containing 2mg/l IAA + 0.1mg/l BAP were excluded because adventitious roots were almost always formed from the callus. In the other eight cultures pure callus was formed. Each culture originated from a single embryo.

For subculture calluses were divided into approx. 4mm cubes, which were transferred aseptically onto fresh medium of the same composition. Cultures were set under dark at $24 \pm 2^{\circ}\text{C}$ temperature.

a-3. Initiation of cell suspension cultures

Suspension cultures were obtained by transferring approximately 0.4g of callus to 25ml of liquid medium in 125ml Erlenmeyer flasks. The cultures were incubated on a horizontal rotary shaker at 120rpm under low light intensity. After one week the cell suspension was filtered through a mesh having pores 100-150 μm wide to

remove large cell aggregates and pieces of callus. The cell number was calculated and an aliquot of 5ml was transferred in 20ml of fresh medium in 125ml Erlenmayer flasks. This time cultures were incubated under the same conditions except that the shaking speed in the rotary shaker was 80rpm. The higher speed of 120rpm was used for enhancing cell separation from calluses.

The media used for secondary cell suspension cultures were the same as the media used for the callus initiation and subculture. All the media were filter sterilized.

Suspension cultures were regularly subcultured by transferring every three weeks a 5ml aliquot to 20ml of fresh medium.

a-4. Measurement of growth of suspension cultures

Growth in cell suspensions was measured by cell counting, determination of the packed cell volume, cell fresh weight, cell dry weight and estimation of the content of DNA. Smith (1973) pointed out that the use of suspension cultures allows a more critical analysis of growth to be made since representative samples may be taken under axenic conditions from a single flask, at various stages in any one sub-culture period and growth parameters can be monitored through a growth cycle.

a-4.1. Cell counting

Cell counting required prior separation of the cells in cell aggregates and this was achieved with a commercial pectinase treatment. 0.1% Rohament P plus 0.2M mannitol were added to the samples (5ml) and the pH was adjusted to 4. Samples were incubated overnight in

the gyrotary shaker (80rpm) at $24 \pm 2^{\circ}\text{C}$ temperature. Next day the cell suspension sample was drawn into a Pasteur pipette and expelled several times. This procedure resulted in separation of cells and their release as single cells in the liquid medium.

For counting the cells a cell counting chamber was constructed in the following way: A microscope glass slide was cut in two pieces which were stuck on another same slide leaving a channel having 0.5cm width and 0.11cm depth. This depth was greater than the diameter of the largest cells and it could well accommodate the suspensions. The channel was covered with a cover slip and by using a Pasteur pipette it was filled with suspension prepared as above and suitably diluted. Cells were allowed to settle and were counted at a magnification of $\times 100$. Twenty random fields were usually counted per channel and five channels were prepared for each sample. At least 10^3 cells were counted per sample and cell number per ml was calculated as follows:

$$\text{Area of the field} = \pi r^2 \text{ cm}^2$$

$$\text{Depth of channel} = 0.11 \text{ cm}$$

$$\text{No. of cells/ml} = \frac{\text{No. of cells per field}}{\text{Area of the field} \times 0.11} \times \text{dilution factor}$$

Calculations of cell number per ml were made every second day from 5ml samples of culture.

a-4.2 Packed cell volume

15ml of culture was transferred in a graduated centrifuge tube and spun for 5 min at approximately $2,500 \times g$ ($\approx 4,000\text{rpm}$) in a bench centrifuge. The volume of the pellet was calculated from the scale on the tube. The results were expressed as $\mu\text{l/ml}$.

a-4.3. Cell fresh weight

Cell fresh weight was determined by collecting the cells on a wet pre-weighed 2.5cm Whatman G.F/A filter supported in a Millipore filter holder. Cells were washed with water to remove the medium, they were drained under vacuum and weighed. The samples taken for determination of this parameter which was expressed in mg/ml were 20ml each.

a-4.4. Cell dry weight

Cell dry weight was determined as described above except that filters were pre-weighed dry. After washing and drawing off surplus wash water, the pads plus sample were dried in a desiccator at room temperature for 72h or until their weight became stable.

Cell dry weight was also expressed in mg/ml and the volume of samples, depending upon the density of the suspension was between 10-20ml of culture.

a-4.5. Estimation of DNA

For DNA determination cells were collected by centrifuging at 2,500 xg for 10 min a sample of 20ml suspension. The collected cells resuspended in water were sonicated for 10 min at 120 W. This time of sonication was enough to break all the cells. The mass which resulted after sonication was collected by centrifugation and either was stored in the freezer or was treated according to the method developed by Setaro and Morley (1977).

DNA was extracted from the sonicated mass of cells at 75°C with 1N perchloric acid (1.5ml) for 30 min. After centrifugation, aliquots 0.1ml of the supernatant solution were used for the assay.

Calf-thymus DNA (Sigma-London) was used for the preparation of DNA standards. Aliquots from a stock solution of calf-thymus DNA (2.5mg/1 in 1N NaOH) were transferred to 10 x 75mm glass tubes to give a series of standard solutions from 25 to 10³ µg of DNA which were dried overnight at 70°C and stored desiccated at 0°C until used. For the assay the DNA standards were treated with 0.1ml of 1N perchloric acid and allowed to stand at room temperature for 10 min to dissolve the DNA. To DNA standards in 0.1ml of 1N perchloric acid for 0.1ml aliquots of tissue extracts of DNA in 1N perchloric acid 0.1ml of an aqueous solution of diaminobenzoic acid - 2HCl (1.32M) was added. The tubes were plugged and incubated in a water bath at 60°C for 30 min. After that the reaction mixture was diluted to 2ml with 0.6N perchloric acid, thoroughly mixed, transferred to 1ml glass cuvettes and read at 420nm.

Each time before a DNA determination was made the calibration curve was prepared from the DNA standards.

- b. Primary cultures
- b-1. Agar cultures
- b-1.1. Isolation of apple embryo cells

Cells were isolated from dormant and after-ripened embryos of apple. Apple seed was after-ripened and sterilized as described in Chapter 4B,b. After the embryo viability was checked by using

the TTC test apple embryos were isolated aseptically in water and then they macerated according to the known procedure i.e. with 1% filter sterilized Macerozyme containing 0.6M mannitol for 2h at 32°C. After post-digestion they were transferred in 5-10ml of nutrient containing 0.6M mannitol and sheared immediately. The resulting cell suspension was poured through a double 63µm mesh to remove any debris, the cell viability was assessed with 0.25% Evan's blue (Gaff and Okong O' Ogloa, 1971) and the cell number was calculated.

b-1.2. Preparation of culture media

Nutrient media were prepared in the same way as described in Chapter 3B. The difference was that instead of Oxoid Agar No. 3, Difco Bacto agar was used to solidify the culture media. Coconut milk was collected from coconuts purchased from the local market and it was stored in the freezer.

b-1.3. Cell plating

The technique followed for plating apple embryo cells was exactly the same as the technique for plating carrot embryo cells. As with carrot, apple embryo cells were plated as evenly as possible on top of an agar layer or were uniformly dispersed within an 1mm thick agar layer. The final concentrations in the culture media were 0.6% agar and 0.3M mannitol. Petri dishes when cool were sealed with Parafilm to avoid desiccation and put in plastic boxes having a wet piece of paper for better control of humidity.

During plating much care was taken for the cells so that they were never exposed to temperatures higher than 35°C during their dispersion in the agar medium. Care was also taken for plating with minimum delay.

The cell density employed for all experiments was 10^4 cells/ml. It was believed that this density would allow enough room for colony development without any overlapping. After plating the cells, Petri dishes were strictly checked for the existence of any cell aggregates. Any Petri dish containing cell aggregates was discarded. Because there were no cell aggregates the plating efficiency was expressed as a percentage of divided cells. The two terms plating efficiency and percentage of divided cells are used in the results interchangeably.

b-1.4. Culture conditions

Petri dishes were incubated at $24 \pm 2^\circ\text{C}$ under dark or light at 200 to 1000 lux because to the light provided by a 40W bulb the diurnal lighting of the room was added.

Observations were made every five days and positions of interest were marked so that the same area could be observed repeatedly. There were five Petri dishes and the results were recorded from ten fields of each Petri dish after one month's culture.

b-2. Liquid cultures

Apple embryo cells of the varieties Phiriki and Golden Delicious were isolated as previously described in agar cultures. The viability of seeds was checked with Evan's blue (Gaff and Okong O'-Ogloa 1971, and after counting the cell number a fraction of 5ml was transferred in 20ml of culture medium to a 100ml conical flask. The cell density was 2×10^5 cells/ml. The conical flasks were incubated in a gyrotary shaker (80rpm) at $24 \pm 2^\circ\text{C}$ under dark or low light intensity conditions, the same as those described in agar cultures.

All the media used in this work contained 0.25M mannitol and were filter sterilized. There were 25 replicates (flasks) per treatment. At least 80 embryos were macerated each time for establishing an experiment with 25 flasks, 25ml medium per flask and cell density 2×10^5 cells/ml.

After a suspension culture was established it could be maintained by regular subculture i.e. by transferring an aliquot of 5ml in 20ml of fresh medium every three weeks.

For the measurement of growth of primary cell cultures the same parameters in the same way as with secondary liquid cultures were determined.

C. RESULTS

a. Derivation of callus and morphogenetic responses

a-1. Embryo development and growth of callus

After-ripened embryo explants started growing from the first day of their culture. Their cotyledons started expanding and moving apart taking finally the positions the leaves have. Under light, chlorophyll formation could be seen by the end of the first day if cultures were set in the morning but it certainly was obvious from the second day. Dormant embryos followed the same pattern of development except that chlorophyll formation started from the third to the fifth day and that with two embryos of the var. Phiriki, cotyledons developed asymmetrically. Under dark (Petri dishes were exposed shortly to light once a week when they were checked for any contamination) cotyledons of the embryos were pale yellow green colour which indicated the formation of chlorophyll.

Under all treatments while the part of the embryo above P.C.A. grew towards a normal plantlet, the part below the P.C.A., i.e. the hypocotyl together with the root, grew to a callus mass. Growth towards callus started from the third day of culture and it was completed within 15 days. All the calluses formed were white or cream in colour and very friable. Table 1 shows that calluses grew bigger under dark and regardless of light conditions calluses on B₅ medium grew bigger. The smallest growth was observed on White's (1963) medium. Between the other two media the bigger growth was observed on Pech et al. (1975) medium.

Table 1
Weights in mg of calluses obtained from apple embryo and hypocotyl explants of the varieties Phiriki and Golden Delicious after 20 days culture under different light conditions.

Explant	Light Conditions	Variety							
		Phiriki				Golden Delicious			
		1*	2	3	4	1	2	3	4
Embryo	Low light intensity	368	539	293	126	390	580	310	110
	Dark	632	811	300	203	580	870	390	240
	Low light intensity	89	88	246	41	70	120	230	70
Hypocotyl	Dark	164	297	453	115	170	310	380	130

* 1: Calluses were derived on Pech et al. (1975) medium;
2: on Gamborg et al. (1968). 3: on Shenk and Hildebrandt (1972) and
4: on White's (1963) medium.

a-2. Morphogenetic responses observed in cultured embryos

Apart from callus formation morphogenetic responses were observed as well in cultured embryos, regardless of physiological status (dormancy) and light conditions. On Pech et al. (1975) medium supplemented with 2mg/l IAA plus 0.1mg/l BAP or with 1mg/l 2,4-D plus 0.1mg/l BAP embryos responded morphogenetically as described in Chapter 4B, a-1 and 4B, g-1, respectively. Fig. 1 shows the response of a dormant embryo after 15 days culture on Pech et al. (1975) medium containing 2mg/l IAA plus 0.1mg/l BAP. This figure in comparison to Fig. 1 of Chapter 4 confirms once more that embryos isolated from dormant seed responded in respect to growth and morphogenesis equally well as the embryos isolated from after-ripened seed.

Morphogenetic responses were very poor on the other three media. Adventitious root formation through callus occurred in a few embryo explants under every medium and formation of structures similar to proembryos described in the previous chapter occurred at the base (petiole) of the cotyledons of a few embryos cultured on White's (1963) and on B₅ medium which containedd respectively 2 and 1mg/l 2,4-D.

a-3. Growth of callus from hypocotyl explants

As mentioned in Materials and Methods hypocotyl explants were isolated from seedlings grown at 4°C from stratified seed. After 70 days of stratification at 4°C apple seed started germinating and after one month seeds had a root up to 4cm long and a hypocotyl 1.5-2cm long. Cotyledons although they were under dark were pale



Fig. 1: Adventitious root formation from callus developed from the hypocotyl and the root of a dormant embryo of apple var. Phiriki, cultured on Pech et al. (1975) medium containing 2mg/l IAA plus 0.1mg/l BAP.

yellow-green and were half covered by the testa. There was no shoot growth.

Callus was formed from every hypocotyl explant. Under dark the whole hypocotyl explant grew to a white callus, while under light callus was formed mainly to the one end of the explant which before isolation was near to the root (Fig. 2a). This polarity of callus formation was shown in 70% of the explants while in the other 30% of the explants either the whole hypocotyl grew towards callus or very little callus was formed in both ends of the hypocotyl. Table 1 shows the weights calluses obtained under different treatments and after 20 days culture. Bigger calluses were obtained with Shenk and Hildebrandt (1972) medium, while callus growth with every medium was better under dark.

In respect with morphogenesis only adventitious root formation occurred in treatments containing IAA (Fig. 2b).

b. Secondary liquid cultures

Secondary liquid cultures were established from calluses derived on the four media employed in this study from after-ripened embryos of the variety Phiriki.

Calluses used for the initiation of liquid cultures were very friable and 10 min after inoculation the liquid medium became cloudy from the released cells. Although these cells were already enough for the establishment of a liquid culture the suspension was filtered after a week. This procedure yielded a higher number of cells allowing more replicates.

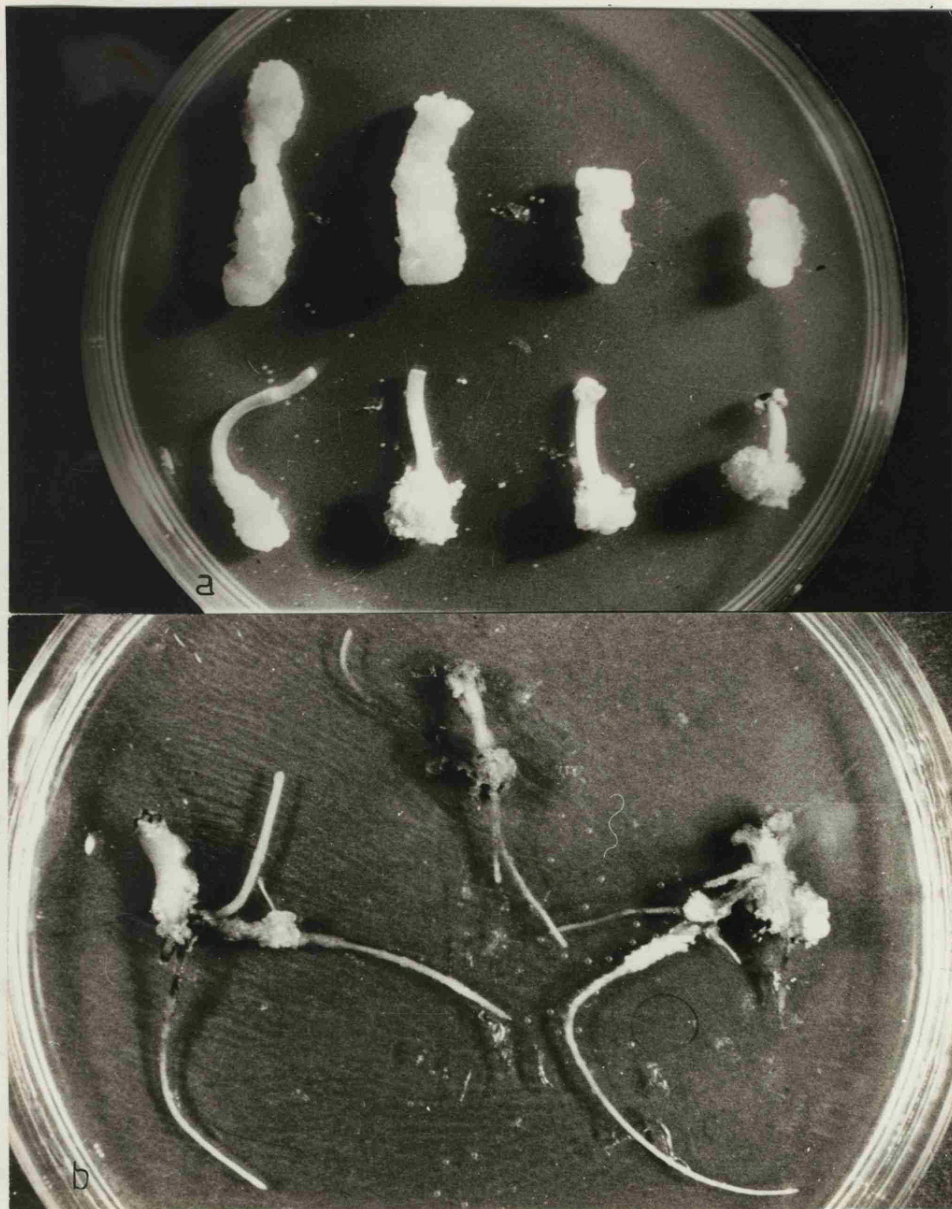


Fig. 2: a. Callus formation from hypocotyl explants of apple, var. Phiriki, cultured on Pech et al. (1975) medium supplemented with 1mg/l 2,4-D plus 0.1mg/l BAP. Under dark (top) the whole hypocotyl explant developed to a callus while under light (bottom) there was a polarity in callus formation which occurred to the end of the hypocotyl towards the root. b. Adventitious root formation from hypocotyl explants of apple, var. Phiriki cultured on the same medium supplemented with 2mg/l IAA and 0.1mg/l BAP. a x 1, b x 1.3.

After filtration, the filtrate consisting of mainly single cells and cell aggregates was used for the establishment of liquid cultures while cell aggregates and pieces of callus which were held on the mesh were resuspended in 25ml fresh medium of the same composition and within two weeks yielded a high number of single cells. The main characteristic though of these suspensions was that formation of big structures occurred like those illustrated in Fig. 3. The frequency of these structures was higher with Pech et al. (1975) and B₅ media and very low with White's (1963) medium. Their size also was big with the first two media and very small with the third. Formation of similar structures has been mentioned by Wallner (1977) in apple liquid cell cultures originated from fruit parenchyma tissue.

Suspension cultures established after filtration followed the same pattern of growth on the four media employed in this study. Diagr. 1 shows the curves for growth parameters for apple cell suspensions of the var. Phiriki in Pech et al. (1975) medium. Similar were the curves of the suspensions grown in the other three media and the values of the growth parameters did not vary significantly with the media. In spite of the similar growth rates of the suspensions which were initiated and maintained on the four media there were some differences. The degree of cell aggregation for example was higher with Pech et al. (1975) and B₅ media while it was very low with White's (1963) medium with which cell suspensions consisted of a few cell aggregates and mainly of freely suspended cells and cells undergoing division. Under all media and in every suspension cell aggregates consisted of small spherical cells while

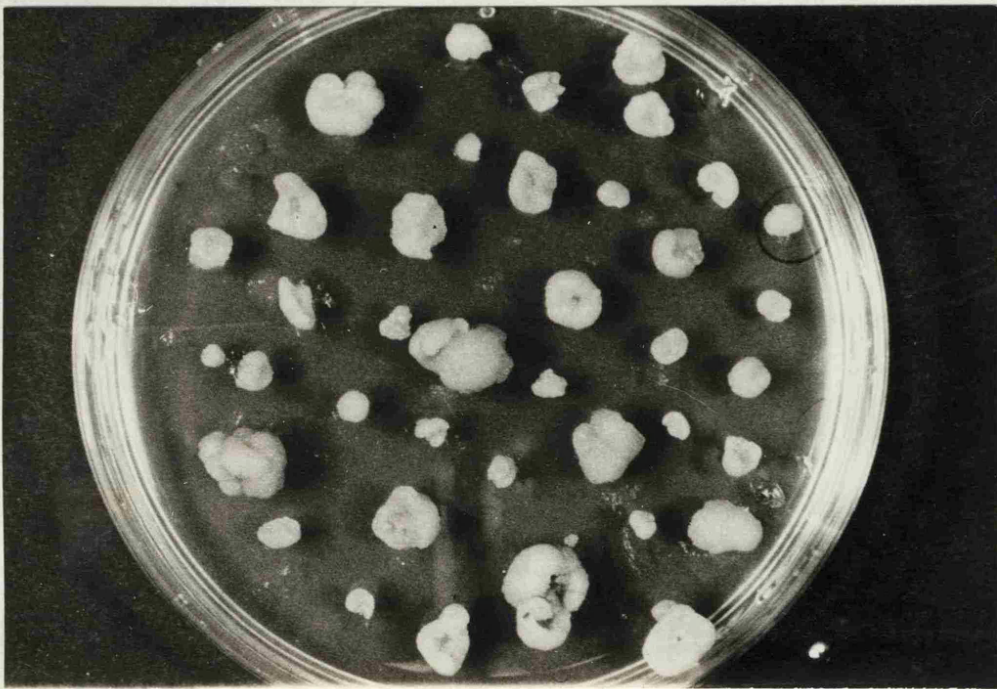
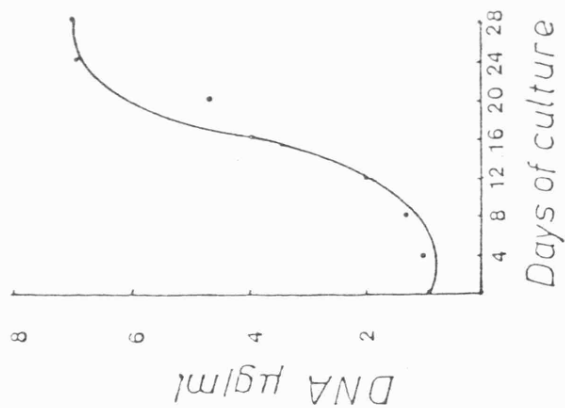
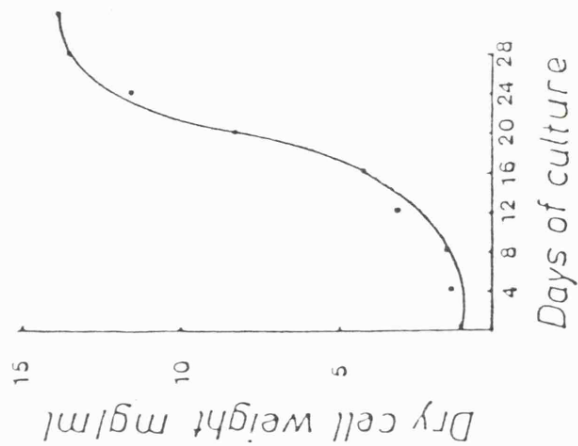
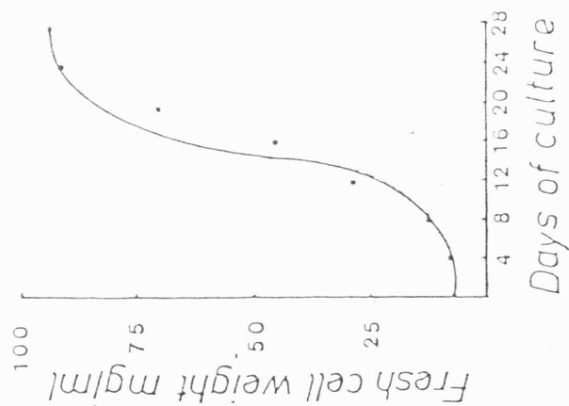
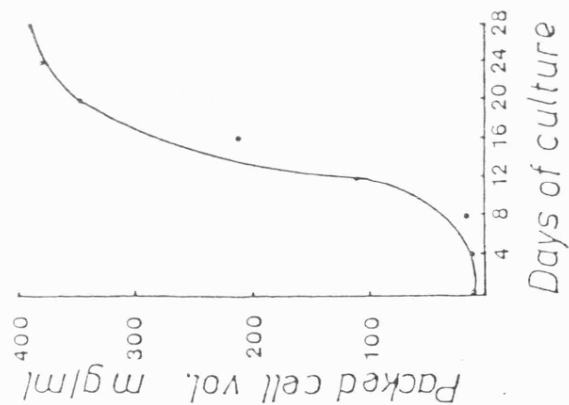
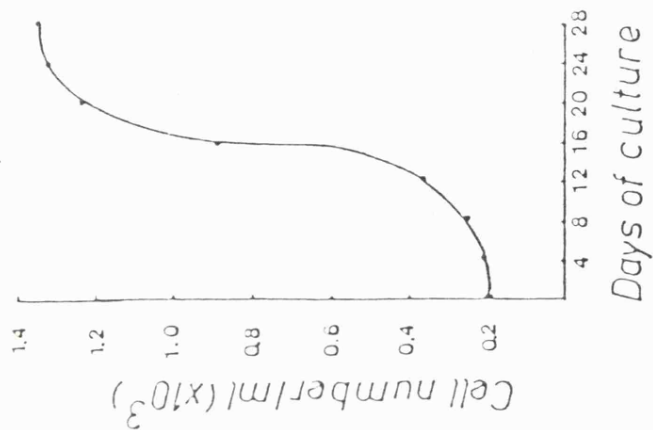


Fig. 3: Multicellular structures which were formed in apple, var. Phiriki, suspension cultures of embryo origin, showing a high degree of cell aggregation.



Diagr. 1: Estimation of cell number/ml, packed cell volume, fresh cell weight, dry cell weight and DNA content in secondary cell cultures of apple var. Phiriki.

the freely suspended cells varied in their morphology and size. Suspensions obtained with White's (1963) medium were dark brown colour. In the other three media the suspensions obtained a lighter brown colour.

If suspension cultures were maintained in the same medium without subculturing then, although the cultures had already entered the stationary phase, cell division continued in some aggregates and after 40 days of culture big structures like those previously described (Fig. 3) were formed. Formation of the same structures also occurred if stock cultures were subcultured every 10-15 days. This is explained by the fact that subculturing at the phase of exponential growth contributes to a higher degree of cell aggregation (Street, 1977a). Again the frequency and the size of these structures were higher and bigger respectively with Pech et al. (1975) and B₅ media while the lowest frequency and the smallest size were observed with the White's (1963) medium which showed the lowest degree of cell aggregation.

b-1. Morphogenetic responses of suspension cultures

Aliquots of the cell suspensions were washed by filtration three times with 20ml medium lacking growth regulators. They were resuspended in media of the same composition (except those from White's (1968) medium which were transferred to the medium of Pech et al. (1975) containing the auxin at the lower level of 0.2mg/l. The level of the BAP and kinetin was kept at 0.1mg/l. The cell density was calculated to be 10^5 cells/ml.

Embryo formation was very rare and it was observed after 40 days of culture only in Pech et al. (1975) medium treatments if either the initial inoculum was from the same medium or from the White's (1963) medium.

For induction of morphogenesis big structures like those illustrated in Fig. 3 were transferred on plates containing 20ml of Pech et al. (1975) medium solidified with 0.8% agar. There were six treatments containing 1) no growth regulator; 2) 0.1mg/l BAP; 3) 1mg/l 2,4-D; 4) 1mg/l 2,4-D plus 0.1mg/l BAP; 5) 2mg/l BAP and 6) 2mg/l NAA plus 2mg/l BAP. The last two treatments were selected because with embryonic tissues gave very consistent morphogenetic responses. For each treatment there were four replicates each one containing at least 10 structures.

All the structures grew towards callus. With the treatment containing no growth regulators growth was ceased after 10 days culture and calluses turned into brown colour. With the other treatments callus growth continued and in all treatments except the one containing no growth regulator and the one containing 0.1mg/l BAP. Calluses after 25 days culture had differentiated green areas. After 40 days culture no morphogenetic response was observed.

In this experiment it was planned for the auxin concentration to be at the lower level of 0.2mg/l. The level of 1mg/l was used by mistake and the experiment was not repeated.

Table 2

Growth regulators and combinations of them with which media were supplemented for inducing embryogenesis in secondary liquid cell cultures of apple var. Phiriki. There were totally four treatments per medium and every treatment had five replicates.

Treatments	Nutrient medium		
	Pech et al. (1975)	B ₅	Shenk and Hildebrandt (1972)
1	No growth regulator	No growth regulator	No growth regulator
2	0.2mg/l 2,4-D	0.2mg/l 2,4-D	0.1mg/l 2,4-D
3	0.1mg/l BAP	0.1mg/l BAP	0.1mg/l kinetin
4	0.2mg/l 2,4-D + 0.1mg/l BAP	0.2mg/l 2,4-D + 0.1mg/l BAP	0.1mg/l 2,4-D + 0.1mg/l kinetin

c. Primary cultures

c-1. Agar cultures

c-1.1. Preliminary experiments

Preliminary experiments were made with each of the four media (Appendix 2) employed in this work to study the effectiveness of auxins (2,4-D, NAA, IAA) and cytokinins (BAP and kinetin) when tested at different levels. Cells were dispersed into the agar medium as evenly as possible and the cultures were incubated under dark at $24 \pm 2^{\circ}\text{C}$. The results were negative since very few cell divisions were observed in each treatment of every experiment. Only in one experiment in which various levels of kinetin in combination with 1mg/l 2,4-D were tested using the Fugimura and Komamine (1975) medium up to four cell divisions observed (Table 3) but the results could not be repeated.

Although the preliminary experiments were unsuccessful from the point of inducing cell division at high percentages and colony formation, they showed that apple embryo cells properly stimulated can divide and form colonies.

Because in the preliminary experiments all the media were supplemented with nicotinic acid, thiamine HCl and pyridoxine as a next step it was decided to study the effectiveness of the same media on inducing colony formation from single apple embryo cells when supplemented with more vitamins.

Table 3

Percentage of apple embryo cells var. Phiriki which divided when plated in agar using the Fujimura and Komamine (1975) medium containing 1mg/l 2,4-D and various levels of kinetin.

a: percentage of cells which showed up to four divisions.

b: percentage of cells which showed one division.

Kinetin concentration mg/l	a	b	Total \pm * SEM
0	1.6	2.7	4.3 \pm 1.8
0.1	1.6	2.9	4.5 \pm 1.0
0.2	2.2	4.6	6.8 \pm 2.6
0.5	4.3	6.9	11.2 \pm 3.6
1	2.8	4.9	7.7 \pm 2.9
2	1.8	4.2	6.0 \pm 1.3
5	0.7	1.2	1.9 \pm 1.9

* SEM = Standard error of the mean

c.1.2. Effect of vitamins

Table 4 shows the vitamins with which each medium was supplemented. The Murashige and Skoog (1962) medium was supplemented with vitamins according to Pech et al. (1975) plus 20mg/l adenine. The Fujimura and Komamine (1975) medium was supplemented with vitamins according to Lin and Staba plus 10mg/l adenine. The B₅ and the Shenk and Hildebrant (1972) media were supplemented with the same vitamins as the Murashige and Skoog (1962) medium.

The growth regulators which were used were 1mg/l 2,4-D in combination with 0.2mg/l kinetin or BAP. Cells were dispersed into the agar medium and the cultures were incubated under dark at $24 \pm 2^{\circ}\text{C}$.

Table 5 shows that a small percentage of apple embryo cells divided up to four divisions. These cells were the smaller 5-10 μm in size and the colonies which consisted of up to 16 cells could not be visually observed. Small was also the percentage of cells which showed one division. These cells were from the smaller as well, i.e. 5-10 μm in size.

Vitamins increased the percentage of divided cells. This increase varied significantly from one medium to another. The higher percentages were achieved with Murashige and Skoog (1962) and Shenk and Hildebrant (1972) media but there was no interaction between vitamins and nutrient media. Single cell clones could not be derived because larger colonies needed to be formed and consequently transferred to other media for further growth and subculture.

Table 4

Vitamins and other organic compounds with which the media were supplemented in order to induce colony formation from single apple embryo cells of the var. Phiriki when plated in agar. The numbers show the concentration of the vitamins in mg/l.

Supplement	Nutrient Medium	
	Fujimura and Komamine (1975)	Murashige and Skoog (1962) B ₅ Shenk and Hildebrant (1972)
Adenine	10	20
Asparagine	-	180
Ascorbic acid	-	50
Biotin	1	0.5
Choline	1	-
Folic acid	1	-
Inositol	5,000	5,000
Nicotinic acid	1	50
Ca-pantothenate	1	50
Pyridoxine	1	50
Riboflavin	1	-
Thiamine	1	50
Thiourea	-	25

Table 5

Percentage of apple embryo cells var. Phiriki which divided when plated in agar media supplemented with vitamins and 1mg/1 2,4-D plus 0.2mg/1 kinetin or 1mg/1 2,4-D plus 0.2mg/1 BAP.

a: percentage of cells which showed up to four divisions. b: percentage of cells which showed one division.

Growth regulators	Nutrient medium											
	Murashige and Skoog (1962)			Fujimura and Komamine (1975)			B ₅			Shenk and Hildebrant (1972)		
	a	b	Total	a	b	Total	a	b	Total	a	b	Total
1mg/l 2,4-D+ 0.2mg/l kinetin	2.4	4.9	7.3	0.9	3.7	4.6	1.8	2.9	4.7	2.1	5.2	7.3
1mg/l 2,4-D+ 0.2mg/l BAP	5.3	6.2	12.5	1.4	3.2	4.6	1.5	3.2	5.7	2.9	5.1	8.0

c-1.3. Effect of coconut milk

Defined media have usually proved inadequate for supporting division of inocula consisting of single cells. Previously it was proved that with single apple embryo cells defined media induced cell division at low percentages, but cells were not capable of continued growth. Coconut milk at different levels was employed in this work to see first if it could promote higher plating efficiencies and second if it could support the continuous growth of cells. Coconut milk and other complex substances like yeast extract are mainly used as a last resort and until they are replaced by defined nutrients. The same media supplemented with the same vitamins as previously described and with 1mg/l 2,4-D plus 0.2mg/l BAP were used. Cultures were incubated at $24 \pm 2^{\circ}\text{C}$.

Table 6 shows that coconut milk at higher levels increased the number of cells which showed more than one division by a factor of approx. 2 (Murashige and Skoog, 1962, and B₅ media) or by a factor of approx. 3 (Fujimura and Komamine, 1975; Shenk and Hildebrant, 1972). It also increased by a factor of approx. 4 the number of cells which showed one division. In spite of its positive effect in increasing the percentage of dividing cells coconut milk did not support continuous growth of cells. Cell colonies which again originated from the smaller cells consisted of up to 16 cells and were not big enough to be transferred to other media for further growth.

Table 6

Percentage of apple embryo cells var. Phiriki which divided when plated in agar media supplemented with various levels of coconut milk, vitamins, 1mg/1 2,4-D plus 0.2mg/1 BAP.

a: percentage of cells which showed up to four divisions. b: percentage of cells which showed one division.

Coconut milk %	Nutrient medium											
	Murashige and Skoog (1962)			Fujimura and Komamine (1975)			B ₅			Shenk and Hildebrant (1972)		
	a	b	Total	a	b	Total	a	b	Total	a	b	Total
0	4.4	6.6	11.0	1.4	5.1	6.5	2.6	4.7	7.3	2.3	6.6	8.9
1	5.7	11.9	17.6	1.8	8.4	10.2	3.9	12.3	16.2	4.2	16.3	20.5
5	7.8	22.6	30.4	4.6	15.8	20.4	5.2	24.6	29.8	5.7	20.8	26.5
10	7.3	26.9	34.2	4.6	23.9	28.5	5.1	20.7	25.8	6.1	25.1	31.2
15	6.1	20.2	26.3	4.9	20.1	25.0	5.8	22.4	28.2	6.1	13.9	30.0

c-1.4. Effect of auxins in combination with BAP

Vitamins and coconut milk increased the plating efficiency in cultured single apple embryo cells in the presence of 2,4-D and BAP. Although the differences between BAP and kinetin with cultured embryonic cells were not significant (Table 3), BAP was used instead of kinetin because apple tissues are more responsive to growth and morphogenesis under BAP than other cytokinins (Chapter 4D). BAP has also been successfully used for the culture of apple cell suspensions originated from fruit parenchyma tissue (Pech et al. 1975; Macheix et al., 1981).

Three auxins 2,4-D, NAA and IAA were tested in combination with BAP for their effectiveness on inducing cell division and colony formation from plated apple embryonic cells. The concentrations of the auxins (1mg/l 2,4-D, 2mg/l NAA and 2mg/l IAA) were chosen arbitrarily. The media were supplemented with vitamins as described in Table 5 and 10% coconut milk. The cells were dispersed into the agar and the cultures were incubated under dark at $24 \pm 2^{\circ}\text{C}$.

Table 7 shows that of the three auxins 2,4-D was most effective on inducing cell division. IAA was the least effective. All the auxins permitted initial divisions of plated cells but none of them sustained continued growth of colonies. It should be mentioned here and in relation to the preliminary experiments that auxins either alone or in combination with a cytokinin could not steadily and at sufficient percentages induce cell division in plated apple embryonic cells if the nutrient media were lacking vitamins and/or coconut milk. To find out if growth regulators were a necessary or absolute requirement for cell division apple embryonic cells were plated in agar media

Table 7

Percentage of apple embryo cells var. Phiriki which divided when plated in agar media supplemented with various auxins in combination with 0.2mg/l BAP. a: percentage of cells which showed up to four divisions. b: percentage of cells which showed one division.

Growth regulators	Nutrient medium											
	Murashige and Skoog (1962)			Fujimura and Komamine (1975)			B ₅			Shenk and Hildebrant (1972)		
	a	b	Total	a	b	Total	a	b	Total	a	b	Total
1mg/l 2,4-D+ 0.2mg/l BAP	8.1	27.4	35.5	4.7	20.3	25.0	5.5	23.7	29.2	5.6	26.7	32.3
2mg/l NAA + 0.2mg/l BAP	6.5	26.2	32.7	4.1	21.1	25.2	4.4	17.8	22.2	3.9	22.6	26.5
2mg/l IAA + 0.2mg/l BAP	3.8	19.8	23.6	2.9	14.6	17.5	1.2	14.1	15.3	2.4	14.8	17.2

supplemented with vitamins and 10% coconut milk and with or without growth regulators.

Table 8 shows that the addition of 1mg/l 2,4-D and 0.2mg/l BAP significantly increased the percentage of divided cells which remained at very low levels in the absence of these growth regulators. The conclusion is that growth regulators were necessary for the induction of cell division in plated embryonic cells of apple. Their effectiveness though could be expressed in the presence of vitamins and coconut milk.

c-1.5. Culture of embryonic cells isolated from different parts of the embryo

From the previous experiments it was shown that up to 35% of plated apple embryonic cells were capable of at least one division and that continued growth of these cells could not be achieved under the experimental conditions employed in this work. On the idea that cells from different parts of the embryo may represent different grades of differentiation, some of which would allow cell division and continued growth of plated cells, cotyledons, embryo axis and intact embryos were macerated separately into single cells and cultured in the Murashige and Skoog (1962) medium supplemented with vitamins (Table 5), 10% coconut milk and 1mg/l 2,4-D plus 0.2mg/l BAP. Cells were dispersed into the agar and the cultures were incubated at $24 \pm 2^{\circ}\text{C}$ under dark.

Although the percentage of divided cells was higher with cells isolated from embryo axis (Table 9), there were no significant

Table 8

Percentage of apple embryo cells var. Phiriki which divided when plated in agar média supplemented with vitamins, coconut milk and with or without 1mg/1 2,4-D plus 0.2mg/ BAP.

a: percentage of cells which showed up to four divisions. b: percentage of cells which showed one division.

Supplements	Nutrient medium											
	Murashige and Skoog (1962)			Fujimura and Komamine (1975)			B ₅			Shenk and Hildebrant (1972)		
	a	b	Total	a	b	Total	a	b	Total	a	b	Total
Vitamins + 10% coconut milk	1.2	5.1	6.3	0.9	4.2	5.1	0.5	7.6	8.1	1.6	8.7	10.3
Vitamins + 10% coconut milk + 1mg/1 2,4-D- 0.2mg/1 BAP	7.6	27.8	35.4	5.1	23.7	28.8	5.4	26.2	31.6	6.8	21.3	28.1

Table 9

Percentage of embryonic cells of apple var. Phiriki which divided when plated in Murashige and Skoog (1962) medium supplemented with vitamins, 10% coconut milk and 1mg/l, 2,4-D plus 0.2mg/l BAP. a: percentage of cells which showed up to four divisions. b: percentage of cells which showed one division.

Cells isolated from	a	b	Total
Embryo axes	7.8	27.9	35.7
Cotyledons	4.9	23.7	28.6
Intact embryos	6.1	26.1	32.2

differences among the percentages of divided cells in respect to the part of the embryo from which cells were isolated. Again cells which divided under every treatment were the smaller, i.e. 5-10 μ m.

c-1.6. Culture of embryonic cells isolated from two varieties

All the previous experiments were carried out using seed of the variety Phiriki. As mentioned earlier seed of this variety was collected from an isolated apple orchard under conditions favouring self-pollination and excess genetic variability was avoided. This experiment is a comparison between cells isolated from embryos of the variety Phiriki and cells isolated from the variety Golden Delicious. Although the conditions under which the collected seeds from Golden Delicious apples were formed were unknown, it is very probable that it was formed in open pollinated flowers and excess genetic variability could not be avoided.

Cells from the two varieties were plated in Murashige and Skoog (1962) medium supplemented with vitamins (Table 4.), 10% coconut milk and 1mg/l 2,4-D plus 0.2mg/l BAP. Cells were plated either in the agar dispersed as evenly as possible or were plated on top of the agar layer. Cultures were incubated under dark and low light intensity as described in Materials and Methods at $24 \pm 2^{\circ}\text{C}$.

Table 10 shows that among the different treatments there were no significant differences although plating efficiencies generally tended to be higher under dark and with the cells dispersed into the agar medium.

Table 10

Percentage of embryonic cells of apple, var. Phiriki and var. Golden Delicious, which divided when plated in Murashige and Skoog (1962) medium supplemented with vitamins, 10% coconut milk and 1mg/1 2,4-D plus 0.2mg/1 BAP under different plating and light conditions. a: percentage of cells which showed up to four divisions. b: percentage of cells which showed one division.

Light conditions	Variety									
	Phiriki					Golden Delicious				
	Cells dispersed in agar		Cells plated on top			Cells dispersed in agar		Cells plated on top		
	a	b	Total	a	b	Total	a	b	Total	
Low light intensity	7.5	29.6	37.1	6.1	26.7	32.8	6.7	25.9	32.6	29.7
Dark	6.3	27.4	33.7	7.1	28.2	35.3	8.1	26.9	35.0	31.4

c-1.7 Culture of cells isolated from dormant embryos

In the previous chapter it was shown that embryonic tissues of apple isolated from dormant seed contain the same morphogenetic capacity in comparison to embryonic tissues isolated from after-ripened seed. Because morphogenetic capacity and organ formation mean first of all capability for cell division it would be reasonable to expect that embryonic cells isolated from dormant embryos would behave in culture similarly in comparison with cells isolated from after-ripened embryos. This experiment is a comparison between cell cultures established from cells isolated from dormant and after-ripened embryos of the varieties Phiriki and Golden Delicious. Cells were dispersed into the agar and the cultures were incubated under dark at $24 \pm 2^{\circ}\text{C}$.

Table 11 shows that embryonic cells of the two varieties isolated from dormant or after-ripened embryos gave no significant differences in respect to plating efficiencies.

c-2. Primary liquid cultures

Cells isolated directly from after-ripened embryos of the varieties Phiriki and Golden Delicious were cultured with Pech et al. (1975) medium containing 1) no growth regulator and 2) 1mg/l 2,4-D plus 0.1 mg/l BAP. There were five replicates per treatment. The cell density was 2×10^5 cells/ml. The medium of Pech et al. (1975) was selected because from the previous work with secondary liquid cultures and primary agar cultures indicated better growth of cells and possibly morphogenetic responses.

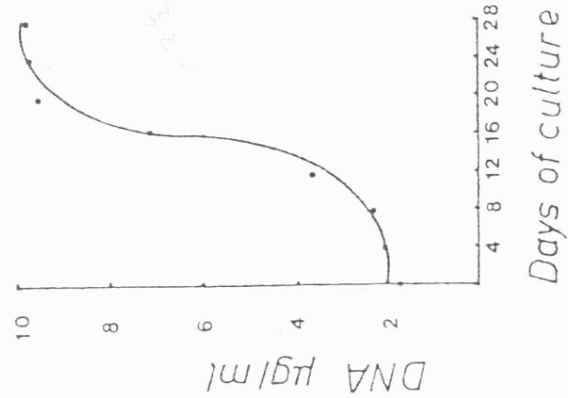
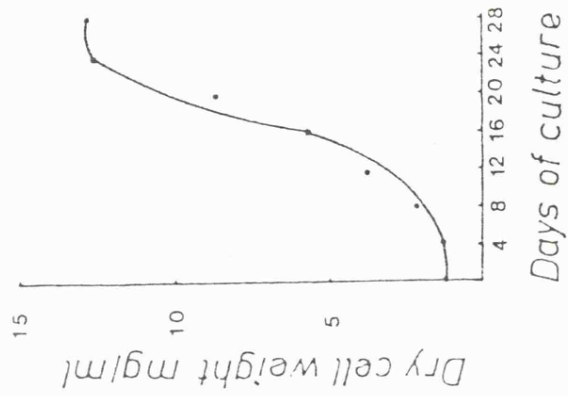
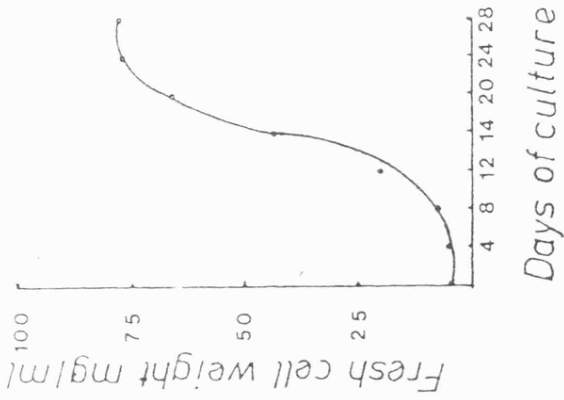
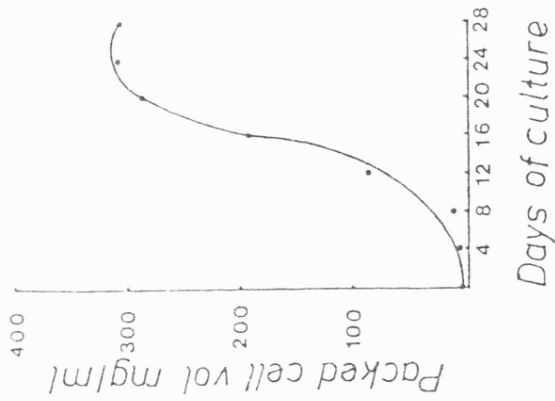
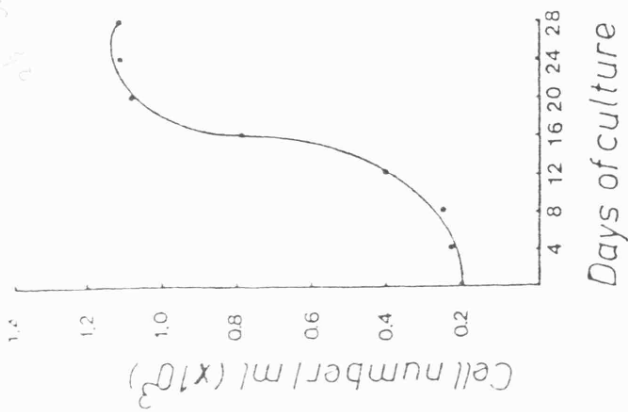
Table 11

Plating efficiencies of embryonic cells of apple var. Phiriki and var. Golden Delicious, isolated from after-ripened and dormant embryos and plated in Murashige and Skoog (1962) medium supplemented with vitamins, 10% coconut milk and 1mg/l 2,4-D plus 0.2mg/l BAP. a: percentage of cells which showed up to four divisions. b: percentage of cells which showed one division.

Cells isolated from embryos	Variety					
	Phiriki			Golden Delicious		
	a	b	Total	a	b	Total
After-ripened	7.8	26.3	34.1	6.7	28.4	35.1
Dormant	6.2	27.5	33.7	8.1	24.9	33.0

Cells from the first days of their culture started changing in morphology. Their granular appearance due to the big internal organelles disappeared gradually, their size was increased and they started dividing. In the medium lacking any growth regulator, cells showed a few divisions and in one replicate formation of one embryo was observed. Diagr. 2 shows the curves of the growth parameters estimated for the var. Phiriki when cells were cultured with 1mg/l 2,4-D plus 0.1mg/l BAP. The growth curves were similar for the same parameter of the var. Golden Delicious.

In determining the growth parameters there was a difficulty in estimating the packed cell volume immediately after cell isolation and during the first eight days of the culture. Cells suspended in the nutrient medium after centrifugation at various times and speeds could not be sunk to the bottom of the tube. Centrifugation at $2,500 \times g$ ($\approx 4,000\text{rpm}$) with the cells suspended in 0.025M CaCl_2 (E. Thomas - personal communication) resulted in sinking of cells to the bottom of the graduated tube except a very small fraction consisting of the smaller cells which were always floating and which were not included in the determination. Before centrifugation cells were collected on a filter and were washed three times with 0.025M CaCl_2 (20ml each time). After the eighth day of culture i.e. after the cells had completed their morphological changes and were actively dividing there was no problem with estimating the packed cell volume with the cells suspended in the nutrient medium.



Diagr. 2: Estimation of cell number/ml, packed cell volume, fresh cell weight, dry cell weight and DNA content in primary cell cultures of apple var. Phiriki.

The inability to collect the cells at the bottom of the tube was not observed only with apple embryonic cells but with embryonic cells of every species used in the work described in Chapter 2. To overcome this problem which led to wrong estimations of the cell number released per embryo (for example the cell number per carrot embryo was estimated between $3-4 \times 10^3$ instead of 15×10^3) it was decided to shear a number of macerated embryos in a properly estimated small volume of 0.6M mannitol so that accurate estimations of cell number per embryo could be made.

A comparison between the growth curves of secondary and primary cell cultures shows that in primary cultures cells divided more rapidly and completed their cycle earlier. The lag phase though was four days in both primary and secondary cell cultures.

DISCUSSION

Isolation of embryos from dormant seeds, followed by culture in light, causes a partial removal of dormancy without cold treatment and results in slow germination and abnormal development of seedlings (Flemion 1934; Côme 1970; Wyzinska and Lewak 1978). The abnormal development of seedlings is related to dwarfism (Flemion 1934, 1956), asymmetric growth of cotyledons (Côme, 1970; Paillard, 1974), perturbations in the appearance of photosynthetic apparatus (Maciejewska et al., 1974) or other metabolic activities (Lewak et al., 1975) Rychter et al., 1970; Tissaoui and Côme, 1975). In this work after-ripened and dormant embryos in culture under light followed the same pattern of development except that chlorophyll formation appeared later in dormant embryos and that asymmetric growth of cotyledons was observed in two dormant embryos.

70% of the hypocotyl explants cultured under light showed polarity with respect to callus formation. Polarity seems to play an important role with living organisms. Before differentiation can be achieved, the cells in the organism must evolve a polarity. Sinott (1960) stated that polarity is the first step in differentiation. Polarity is evident early in the bipolar development of the embryo from the zygote and it is later expressed in the external and internal organisation of the plant into the root and shoot. It is also evident in various phenomena at the cellular level. According to Esau (1965) an illustration of polar behaviour of individual cells within the plant body is the unequal division resulting in physiologically morphologically different cells. As an example for this she reported that in epidermis of certain roots after an unequal

division only the smaller cell produced a root hair. Polarity is exhibited not only by the plant as a whole but also by its parts, when these parts (either they are called explants or cuttings) are isolated from the plant. Vöchting (1878) studied the polarity of regeneration in plants and he found that stem tissue is strongly polarized. He also concluded that the intensity of polarity effect varied considerably among stems, roots and leaves. Stems showed strong regeneration polarity, roots showed somewhat weaker and much weaker regeneration polarity was shown by leaves. Vöchting's results are commonly observed in leaf cuttings where roots and shoots arise at the same position, usually the base of the cutting showing that there is little or no polarity influence. Polarity is also exhibited in grafting and in tubes and tuberous roots (Hartmann and Kester, 1975).

The nature of polarity is ubiquitous and not surprisingly polarity effects have been observed in vitro in the culture of inflorescence stalk sections from Gladiolus (Ziv et al., 1970), Asparagus (Takatori et al. 1968) and on stem segments of Rhododendron (Pierik and Steegmans, 1975). Abbot and Whiteley (1976) increased the percentage of rooted apple shoots from 30 to 80% by placing the IBA treated shoots in an inverted position on the medium while in work described in Chapter 4 the position of cotyledons did not affect organogenesis. Polarity effects have been shown to affect organogenesis from bud-scale explants of Lilium longiflorum (Hackett, 1969). The greatest organogenetic potential occurred from the basal portion of the bulb-scale and along the abaxial edge of the explant.

The polar formation of callus in hypocotyl explants of apple observed in this work is another exhibition of polarity in vitro. The fact that this polarity was observed only when hypocotyls were cultured under light should be explained as follows. Hypocotyl explants were isolated from seedlings grown in the refrigerator under dark. After isolation those explants cultured under dark grew into callus, because their exposure to light was too short to cause any further differentiation preventing cell division. Those explants which were cultured under light differentiated chlorophyll, as hypocotyls of seedlings did, and callus formation occurred at the one end obviously from cambial tissue. The fact that callus was formed mainly at the one end should be attributed to a growth regulator (probably auxin) gradient interacting with the growth regulators of the medium with or alternatively, which is less probable to an anatomic differentiation gradient.

It was very easy to obtain calluses from apple embryo explants of the two varieties. According to Gamborg and Shyluk (1981) the growth of callus is influenced by several factors. Among them is the initial pH of the medium optimal between 5.5-5.8 and the temperature optimal between 26-28°C. Light is generally not essential and cultures behave equally well under light and dark. In this work the initial pH of the media and the temperature were within the limits described as optimal by Gamborg and Shyluk (1981). In respect to light conditions however the growth of calluses was greater under dark. Chong and Taper (1974) found that apple stem callus var. Cortland grew better in the dark, whereas growth of callus of the var. Robusta was not influenced by light. A detailed review about the

influence of light on callus cultures is given by Seibert and Kadkade (1980).

Cells of embryo origin grew well in secondary suspension cultures. The White's (1963) medium supported better the growth of cells than tissues. The other three media supported the growth of cells and tissues equally well. The pattern of growth of cells of embryo origin is similar to the pattern of growth of cells of fruit parenchyma tissue (Pech et al. 1975). Similar was the pattern of growth of cells of embryo origin in primary liquid cultures. The difference was that with primary cultures the growth rate tended to be faster and the growth cycle was completed earlier in comparison to secondary cultures. The DNA content was higher with primary cultures. This was due to the embryogenic nature of the cells and their direct isolation from the embryos.

These experiments failed to induce embryogenesis in liquid cell cultures of apple by following the sequential effect, i.e. by transferring the cultured cells to media lacking the auxin or containing it at a lower concentration. The fact however that sporadic embryo formation was observed indicated that a more systematic and detailed investigation might reveal what the specific requirements (environmental, nutritional, cultural) are for regularly inducing embryogenesis in apple cell suspension cultures. When transferring the inoculum from the high to low auxin or to medium lacking the auxin for inducing embryogenesis low cell densities should be tried. It is probable that high cell densities favour cell growth while with low cell densities, cells are directed towards embryogenesis. In this work the cell density of 10^5 cells/ml might

have been high enough to prevent embryogenesis.

Cells grew better in agitated liquid cultures in comparison to agar cultures. With agar cultures defined media used in this work failed to induce cell division at high percentages. The use of coconut milk increased the number of the divided cells but failed to support their continued growth. Cells which had divided were the smaller (5-10 μ m) and from the figures of Chapter 4Bg it is obvious that the smaller cells are the epidermal. Although a report of 100% plating efficiency with tobacco cells (Gibbs and Dougall, 1963) suggests that virtually every cell is capable of division the more usual observation has been that only part of a plant cell population divides and produces colonies (Bergmann, 1960; Blakely and Steward, 1964; Jones et al., 1960; Kato and Takeuchi, 1963; Muir et al., 1956, and Torrey and Reinert, 1961). In this work this part of the cell population originated from the embryonic epidermis.

From the three auxins tested 2,4-D was the most effective and IAA was the least effective. Similar results were obtained by Earle and Torrey (1965) with Convolvulus cells plated on defined media, who found that 2,4-D was more effective than NAA and NAA more effective than IAA.

No macroscopic group of cells, i.e. macroscopic colony, was derived from apple embryo cells plated in agar media. Earle and Torrey (1965) reported that isolated single cells have shown quite exacting nutritional requirements. In this work the nutrient media used may not respond to the exact requirements of apple embryo cells. Apart from the chemical composition of a medium, however, other factors may affect cell division and colony formation. Earle and Torrey

(1965) for example, found that the level of the pH after autoclaving affected the type of colonies formed from Convolvulus cells.

Bergmann (1960) and Torrey and Reinert (1961) reported that cells plated on media lacking coconut milk or yeast extract were capable of a few divisions but not of continued growth. In these experiments coconut milk increased the percentage of divided cells but it did not support the continued growth of cells.

There were no significant differences between cultured cells isolated from the var. Phiriki and Golden Delicious (Table 8). The percentage of cells which divided did not vary significantly with the variety and with the type of culture. In contrast with carrot embryo cells apple embryo cells either dispersed in agar or plated on top of an agar layer, showed no significant difference in the percentage of divided cells. In respect to light conditions between cells cultured under dark and low light intensity there were no significant differences. These results do not agree with the results obtained by Earle and Torrey (1965) who found that low intensity (200 ft-c) of white light severely inhibited division of plated Convolvulus cells.

Gibbs and Dougall (1963) failed to obtain clones from tobacco cells plated on a defined medium which supported growth of tobacco callus. From this work it is evident that clones from apple embryo cells could not be obtained with defined media which supported growth of apple callus. It is possible that the media which could probably support growth of apple embryo cells are different from the media supporting apple callus growth.

The single cell nature of the agar cultures should be considered as the main reason for the failure of apple embryo cells to grow. Cells which are in organic contact in a group have a higher probability of growing than free single cells do and they seem to have a probability of growth which is vanishingly small. This is because single cells are deprived of those conditioning factors which may be provided by the cell aggregates. These requirements of single cells can be satisfied with the use of conditioned media. Blakely and Steward (1964) reported that free carrot cells divided much more frequently when the cells were plated in conditioned coconut milk media and when carrot root explants were placed on the medium near the cells. More about the use of conditioned media and about conditioning of media has been reported by Street (1977)a.

GENERAL DISCUSSION

A method is presented in this thesis for isolating single, morphologically intact and viable embryonic cells at densities high enough to start primary cultures. This method seems to have made applicability, and in combination with cellulase treatment it can provide a means for the preparation of protoplasts from embryo cells.

Primary cell cultures consisting of single cells and showing high morphogenetic capacity, like those obtained by macerating carrot embryos present advantages over callus, secondary cultures and protoplasts for obtaining solid variants or mutants. The major disadvantage of calluses are the relatively slow growth rates and the possibility of masking of mutant cells by non mutant cells which survive in the presence of inhibitory compounds because of the mass of the callus (Tomes and Swanson, 1982). The problem similar is with secondary cultures which show the disadvantage of cell aggregation (Melchers and Bergmann, 1959; Tulecke 1966; Liau and Boll, 1971). Cell aggregation creates difficulties in selecting variant or mutant cells from a population since the aggregate chimerism often obscures the trait. There are similar problems with protoplasts in liquid cultures, where they have ^a tendency to aggregate, grow in contact and fuse together (Lawrence, 1980).

The embryonic cell isolation method constitutes a starting point for the study of problems concerning primary cultures like the control of the environmental conditions and the uniformity of such cultures (Thomas and Davey, 1975b). According to the authors very little is ^k known of the extent to which the behaviour of cells in primary cultures can be controlled by adjustments to their physical and chemical environments. The results

obtained by culturing embryonic cells of carrot and apple and presented in this thesis are a contribution in this direction. Two other objectives in respect to primary cultures are to determine their uniformity in response to morphogenetic compounds and to compare this response with the response of more highly differentiated cells (Thomas and Davey, 1975b). Carrot embryonic cells responded uniformly to morphogenetic compounds and apple embryonic cells gave similar growth curves for each parameter in comparison to secondary liquid cultures of embryo origin and in comparison to secondary liquid cultures of fruit parenchyma origin (Pech et al., 1975).

Embryonic cells isolated from zygotic carrot embryos proved to be of high morphogenetic capacity and approximately 30% developed directly to embryoids which reached different stages of development. According to Halperin and Wetherell (1964) the further development of cell-culture derived embryoids to plantlets depends on the constitution of the nutrient medium. Embryonic tissues of apple were also of high morphogenetic capacity, showing easy plant regeneration, but with apple embryonic cells repeatable plant regeneration could not be obtained.

The ability to obtain whole plants from single cells of fruit cultivars is of special interest for the plant breeder and possibly for the plant propagator. The apple embryo cell culture in this work represented an unsuccessful attempt to induce any morphogenesis in agar and liquid cultures. In agar cultures even colony formation could not be induced. The fact though that sporadic embryo formation occurred in heterogenous liquid cultures indicates that embryogenesis is possible and the key for its success may lie with the correct combination of medium components and environmental conditions. This view is further supported by the striking morphogenetic responses of the embryonic ^tissues.

As emphasized in Chapter 1, dependable plant regeneration from cell cultures of many species is the major limitation of in vitro selection for plant improvement. Apple could possibly be improved through somaclonal variation only if it were amenable to in vitro manipulation for the growth of cell cultures and for the easy regeneration of plants from variant cell cultures. In this thesis the exploratory work on apple regeneration using embryonic tissues was successful but with apple embryo cell cultures, based on the carrot model and on the exploratory work with embryonic tissues of apple, plant regeneration could not be obtained. So research effort has yet to be focused on the regeneration of apple from embryonic cell cultures first and then from cell cultures isolated either from mature clonal material or from experimentally induced somatic embryos. As a next step towards repeatable plantlet regeneration an investigation should be made using cell cultures originated from the petiole of cotyledon explants, because petiole tissue of cotyledon explants has been proved to be very responsive to morphogenesis.

Instead of drawing information from a successful regeneration study with apple embryonic cells (isolated from seed) and using it for a regeneration study with mature clonal material, as aimed in this work, another alternative could be followed. Somatic embryo formation could be induced experimentally in clonal tissue and then primary cell cultures could be established by macerating somatic embryos. Primary cell cultures derived from experimentally induced somatic embryos might be more useful for establishing a method for repeatable plant regeneration and consequently for in vitro selection procedures via somaclonal variation.

So far there is very little research work towards experimental

induction of somatic embryogenesis in apple. In this work embryo formation was induced on cotyledon explants cultured on media containing 2,4 - D. Perhaps under similar experimental conditions embryo formation could be induced in clonal material such as young leaves;

In the process of macerating zygotic embryos it was found that plant embryos are surrounded by a cuticle. The same applies to adventive embryos. The discovery of this cuticle that plant embryos differentiate adds to the idea that a cuticle is an important prerequisite if living ^tfissues are to be maintained at some elevation above the surface of the land and raises the question as to when this cuticle originates i.e. at what stage of the zygotic and adventive embryo development. Konar and Nataraja (1965) reported that each globular embryoid developed from cell suspensions of Ranunculus sceleratus grown in agar was surrounded by a "common membrane" indicating its origin from a single cell. They did not mention anything though as to when this "common membrane" appeared and how this "common membrane" indicated the single cell origin of the embryoids.

Thorpe (1982) reviewed the requirements for organized development. Among them one view proposed by Steward and his coworkers (1958,1964) as a requirement for organized growth and development is that physical and/or physiological isolation of a cell from the correlative influences of other cells in a tissue system or organized structure is a prerequisite for a cell to express its developmental potential. This view is supported by the work of Button et al., (1974) and Kohlenbach, (1977) in which thick cell walls have been observed around meristematic cells which form embryogenic clumps suggesting a possible physiological isolation of these cells and by the work of Danilina (1972) who demonstrated that in the early phases of embryogenesis, individual cells

of the callus tissue are isolated from the surrounding tissue which forms a thick envelope.

Thomas and Davey (1975b) commenting on the single cell origin of adventive embryos suggested that the direct development of a single cell to an embryoid without an intervening callus phase could possibly be achieved by plasmolysing an isolated cell and inducing the plasmolyzed protoplast to regenerate a new wall within the existing cell wall. The original wall would have a barrier effect to the freely dividing cells and would restrict organized divisions to the internal regenerated cell, leading to embryogenesis. These investigators based their suggestion on the observation that a developing multicellular microspore in culture or a single dividing epidermal cell of Ranunculus show a common feature; they undergo divisions within the existing cell. They also correlated the original cell wall, which would act as a barrier to the free proliferation of cells and which would restrict organized development, with the exine and intine of a microspore, both described as sporoderm.

If the previous observations are related 1) To the fact that plants possess the additional feature of separate, asexually produced cells, called spores, capable of development, which is often independent of the plant, or may be enclosed within it 2) To the fact that, in many lower organisms the zygote becomes a free living spore which before it is freed forms a membrane around itself and 3) To the possibility that the cuticle surrounding the aerial parts of a plantlet may be an evolutionary type of the membrane of the zygote, the conclusion which can be drawn is that it would be worth to investigate at what stage of the somatic embryogenesis the cuticle appears and what is its probable significance for somatic embryogenesis.

In this work the cells which developed to embryoids were the smaller. Observing carrot and apple embryos it was found that the smaller embryo cells were from the epidermis and during enzymic maceration, these cells were separated from the cuticle. After isolation and during culture these cells were presumably the most suitable in either immediately differentiating a cuticle around themselves or in transmitting the signal for its differentiation during early embryonal development.

Embryogenesis from epidermal layers of various explants has been reported (Mc William et al., 1974; Kato, 1966). The responsiveness of the epidermal cells to embryogenesis and other types of morphogenesis like adventitious bud and root formation may possibly be either because of their ability to form a cuticle around themselves or because of the already existing cuticle of the explant. Cuticles (Martin and Juniper, 1970) are known to control physiological activities. These activities such as the gaseous exchange in combination with their protective mechanisms may favour embryogenesis from epidermal cells. This idea is supported by the observation (not mentioned in the results) that in cultures of cotyledon explants of apple var. Phiriki the dividing cells which formed the adventitious organ did not break the continuity of the cotyledonary cuticle. The cuticles of the adventitious organs (embryos or shoots) and of the cotyledon explant were continuous.

Embryogenesis in cell cultures commences from the smaller and isodiametric cells than from the large and very highly vacuolated ones (McWilliam et al., 1974; Jones, 1974). Moreover, according to Steward et al., (1966) the embryogenetic responses of cultured cells may partly depend on their "prior history" i.e. on the part of the

plant body from which they have been derived and in the case of cultivated plants on the genetics of the strain. In this work on the embryonic cells isolated from zygotic embryos the smaller and more or less isodiametric cells developed directly to embryoids and this is in agreement ^swith the results obtained by the previous investigators..

In the past Kato (1968) and Fujimura and Komamine (1980) reported on the serial observations of the adventive embryogenesis observed in tissue and cell cultures respectively. Their systems though presented certain disadvantages like the existence in cell cultures of cell aggregates from which the adventive embryos arise or the initiation of embryoids in multicellular hypocotyl explants. The observation of single cells developing to embryoids without an intervening callus phase constitutes a very useful system for studies concerning the resemblance between the zygotic and adventive embryogenesis.

As in the case in zygotic and adventive embryos at different stages of their development, it is likely that meristems and apomictic embryos can be macerated by following the same procedure. Maceration of adventive haploid cells will give a new direction to the study of the culture of haploid cells, since pollen culture is difficult.

Maceration of zygotic and of adventive embryos would provide useful information on the cell number per zygotic embryo and adventive embryo, on the cytology of the cells which zygotic and adventive embryos consist of and about the morphogenetic capacity of these cells after isolation. Furthermore it could provide useful information on the

inheritance of genetic stability from a zygotic to an adventive embryo through a single isolated cell.

Since maceration of zygotic and adventive embryos is possible it would be interesting to induce embryo formation from single zygotic embryo cells, then macerate the adventive embryos into single cells and try to induce again embryogenesis in them and so on. Such a study would provide information on whether the morphogenetic capacity of embryonic cells is firmly connected with the embryonic (adventive and zygotic) nature of the cells or whether the morphogenetic capacity is connected only to their zygotic nature. In addition to that, adventitious embryos formed in tissue or cell cultures of different origin could be macerated into single cells and the morphogenetic capacity of these cells could be related to the original explant if this capacity is not closely connected with their adventive embryonic nature. If embryonic cells isolated from zygotic and from adventive embryos show the same morphogenetic potential, because zygotic embryo isolation is not a convenient process batches of somatic embryos representing a certain genotype or clone could be used for the study of various aspects including propagation. These batches e.g. 500 carrot embryos/gr callus/month (Murashige 1977) would provide abundant material under aseptic conditions at any time of the year.

Because tissue cultures could be used to isolate and propagate (by cloning procedures) some somatic mutations of natural occurrence or induced by radiation (Handro, 1981) maceration of carrot embryos and the following development of the single cells to embryos could constitute a useful system for the study of somaclonal variation and for the induction of mutations. Zygotic or adventive embryos could be irradiated before cell isolation and then during culture, cells

could be subjected to the in vitro selection procedures in order to isolate mutant cell lines or individuals. Another approach would be to irradiate the adventive embryos originated from single cells isolated from a single embryo and then induce their development to plantlets. Nitsch (1969) and Nitsch et al., (1969) obtained different phenotypes of tobacco plants, after irradiation of plantlets produced by anther culture. Similar results were obtained by Corduan (1975) working on callus and plantlets from anther culture of Hyoscyamus niger.

Embryonic tissues of apple of the var. Phiriki and Golden Delicious isolated from dormant and after-ripened embryos were found to be very active morphogenetically. Approximately 60 adventitious shoots were produced per single apple embryo and half of them developed to rooted plantlets. It is almost certain that such plants of a single embryo origin can easily be propagated by the means of rapid shoot multiplication (Lane, 1982) and produce large populations of cloned individuals in a relatively short time.

The cultivated fruit varieties are raised mainly on clonal rootstocks but seedlings still possess an important place at least for some applications with peach, almond, plum and pear (Hartmann and Kester, 1975c; Rom, 1983, OKie, 1983 and Westwood and Lombard 1983). Clonal rootstocks present two advantages over the seedling stocks. The first is the uniformity both of the stocks themselves and of the trees raised on them. The second is the control of the vigour and precocity of the tree which becomes possible by using selected clonal stocks of known performance. The disadvantages connected with the use of clonal rootstocks, are that they are relatively costly to propagate and that in time they are liable to accumulate viruses

which affect the vigour of the stocks themselves and produce symptoms in the varieties budded or grafted on them.

The advantage of seedling -stocks are that they are cheap and virus free and the disadvantage is their heterogeneity. To overcome problems related to heterogeneity and lack of uniformity in apple seedling -stocks efforts were made to produce seedlings at commercial scale from apomictic embryos (Sax, 1959, Luckwill and Campbell, 1954; Campbell, 1960 and Campbell and Wilson, 1961). In Greece (Deciduous fruit tree Institute, Naoussa-personal communication) wild peach trees the seed of which produces uniform and disease resistant seedling-stocks have been selected.

The method of cotyledon and axis culture developed in this work is a possible answer to the production of cloned (of single embryo origin), uniform, virus free and cheap seedling - stocks from every fruit or ornamental cultivated variety, rootstock clone and virtually every individual apple tree forming seed, as well as from other fruit species. This method is connected with some additional advantages related to tissue culture, i.e. independence of weather conditions, mass propagation, transfer under aseptic conditions and at large quantities in a relatively small volume, storage of the produced stock material etc. Furthermore in relation to apomixis it can be used as a means of asexual propagation. In this case a method for rapid shoot multiplication of the plantlets produced from a single embryo may not be necessary because many apomictic embryos can be used instead of one embryo.

Apart from the production of rootstock material the methodology of cotyledon culture may possibly be used for the mass propagation of

scion cultivars of apple, leaves being homologous organs, could be isolated and cultured at any early stage of their development so that they produce similar morphogenetic responses. Therefore the culture of young leaves isolated either from trees grown in the field or from tissue culture plantlets should be attempted under the conditions which favour plantlet production directly from cotyledon explants. P

Given the uniform morphogenetic responses of heterogenous zygotic embryo explants of two varieties of apple the conclusion is that within the species the expression of the morphogenetic capacity is strongly related to the embryonic nature of the explant and to the degree of differentiation of the cells because in case of cotyledon culture only a part (petiole) of the embryonic explant responded morphogenetically.

Skirvin (1981) commenting on fruit improvement through tissue culture methods, reported that the usefulness of mutation induction to improve particular clones is limited because of the inability to either recognise or separate fruit crop chimeras into pure types. He suggested that this inability could be avoided by growing shoots or even complete plants from single cells because if a mutation occurs in a single cell the resulting individual will be either completely normal or completely mutant in genotype. Because mutagenic treatments tend to produce a proportion of deleterious mutations the hope is that somaclonal variation arising from spontaneously variable cells in the cultures will be more useful. The potential usefulness of somaclonal variation has been demonstrated. Variation in morphological (Liu et al., 1972) and disease resistance traits (Nickell and Heinz, 1973; Heinz et al., 1977; Larkin and Scowcroft, 1981) has been observed amongst sugar cane somaclones. Shepard et al., (1980) claimed to have demonstrated that it could be simpler to selectively improve popular potato varieties

than to create new ones and Larkin and Scowcroft (1981) gave substantive and extensive examples of somaclonal variation in cultured subclones and in regenerated plants (somaclones). ✓

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Appendix 1

Composition of the Nitro Blue Tetrazolium incubation medium; after Hauser E.J.P. and Morrison J.H., Amer. Journ. Bot. 51 (7) : 748-752, 1964.

17 ml 0.06M Sorensen's phosphate buffer (pH 7.4)

17 ml 0.2M Sodium succinate (0.066M in total medium)

17 ml Nitro Blue Tetrazolium (1mg/ml)

12.65 mg Sodium amytal (1mM in total medium)

Appendix 2

Composition of media used for plant tissue and cell culture.

	1 [*]	2	3	4	5	6
Macronutrients	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
NH_4NO_3	-	1650	1650	-	-	-
NH_4Cl	267	-	-	-	-	-
KNO_3	5560	1900	1900	2500	2500	80
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	219	440	440	150	200	-
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	185	370	370	250	400	720
KH_2PO_4	68	170	0.85	-	-	-
$(\text{NH}_4)_2\text{SO}_4$	-	-	-	134	-	-
$(\text{NH}_4)\text{H}_2\text{PO}_4$	-	-	-	150	300	-
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	-	-	-	-	-	16.5
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	-	-	-	-	-	300
KCl	-	-	-	-	-	65
$\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$	-	-	-	-	-	200
Micronutrients						
KJ	0.375	0.83	0.83	0.75	1	0.75
H_3BO_3	2.4	6.2	6.2	3	5	1.5
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	9.2	22.3	22.3	-	-	7
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	-	-	-	10	10	-
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	4.05	8.6	8.6	2	1	3
$\text{NH}_4\text{M}_2\text{O}_{24} \cdot 4\text{H}_2\text{O}$	0.0925	-	-	-	-	-
$\text{Na}_2\text{M}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$	-	0.25	0.25	0.25	0.1	0.00016
M_2O_3	-	-	-	-	-	0.0001
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	-	0.025	0.025	0.025	0.2	0.001
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	-	0.025	0.025	0.025	0.1	-

$\text{Fe}_2(\text{SO}_4)_3$	-	-	-	-	-	2.5
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.0278	27.8	27.8	27.8	15	-
	+	+	+	+	+	
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	0.0372	37.3	37.3	37.3	20	-
Vitamins						
Meso-inositol	-	100	100	100	1000	100
Nicotinic acid	5	-	0.05	1	5	0.5
Pyridoxine.HCl	0.5	-	0.005	1	0.5	0.1
Thiamine.HCl	3	0.1	0.01	10	5	0.1
Glycine	-	-	-	-	-	3
Ascorbic acid	-	-	50	-	-	-
Thiourea	-	-	25	-	-	-
Asparagine	-	-	180	-	-	-
Ca-Pantothenate	-	-	1	-	-	-
Biotine	-	-	0.01	-	-	-
Sucrose	2%	3%	3%	3%	3%	2%
pH	5.4-5.5	5.7-5.8	5.7-5.8	5.5	5.9	5.8

- * 1: Fujimura, T. and Komamine, A. (1975) medium: Pl. Sci. Letts.
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